# RESISTANCE OF THE ZEBRAFISH (*Brachydanio rerio* Hamilton-Buchanan) TO ZINC SULPHATE

by

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This thesis is the product of my own original research, except where specifically stated to the contrary.

Sections 2, 3 and 4 are based on the following three papers, respectively:

Skidmore, J.F. 1964. Toxicity of zinc compounds to aquatic animals with special reference to fish. Quart. Review Biol., 39: 227-248.

Skidmore, J.F. 1965. Resistance to zinc sulphate of the zebrafish (Brachydanio rerio Hamilton-Buchanan) at different phases of its life history. Ann. Appl. Biol., in the press.

Skidmore, J.F. Resistance to zinc sulphate of unhatched zebrafish embryos (Brachydanio rerio Hamilton-Buchanan) after removal or rupture of the chorion. Submitted for publication.

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# CONTENTS

# 1. INTRODUCTION

Numerous investigators have examined the toxicity of poisons to aquatic animals. Much of the literature can be traced through reviews by Doudoroff and Katz (1950, 1953), Jones (1957, 1964), Hynes (1960), Lloyd (1962), and Skidmore (1964). Most frequently, samples of from two to ten small fish have been used as test animals in toxicity bioassays. With few exceptions, the results of such bioassays form the only available indication of the concentrations of poison which can be tolerated by a natural population of aquatic animals. Much of this work has been concerned with environmental factors which modify the toxicity of a poison to a particular organism, rather than with factors which influence the resistance of the test organism.

The resistance of an aquatic animal to a poison may change as a result of a number of factors, particularly the development of a new phase in the life cycle, the acclimatization or adaptation of the animal to changed conditions, and the development of a resistant strain from the survivors of selective mortality.

If an animal species is to complete its life cycle in a given body of water, it must be able to withstand any adverse condition in the environment - such as the presence of a poison - at each stage in its life history. Hynes (1960, p. 74) emphasized the necessity for bioassays at the most susceptible stage of the life cycle of the test organism, yet no-one has hitherto studied how the resistance of any aquatic animal to any poison varies with life history. This thesis reports on the resistance of the various stages of the life cycle of a particular species (the zebrafish, Brachydanio rerio Hamilton-Buchanan) to a particular toxin (zinc sulphate), thus identifying the most susceptible stages.

Zinc sulphate was selected for study because zinc compounds are common industrial wastes in many parts of the world, including the Canberra region, and are therefore of importance in pollution ecology. The literature on the toxicity of zinc compounds to aquatic animals is extensive. Much is known about the effects of the dilution water on the toxicity of zinc and this knowledge was considered to be a good basis for further work. The literature relevant to the present study will be summarized in Section 2.

## 2. REVIEW OF THE LITERATURE ON THE TOXICITY OF ZINC

### a) Lethal concentrations of zinc

The toxicity of zinc compounds has been chiefly studied with reference to two groups of animals - mammals and fish. The literature on mammals has been reviewed by Underwood (1956) and Vallee (1959, 1962) and will not be discussed here. The early literature on fish has been briefly reviewed by Doudoroff and Katz (1953) and Doudoroff (1957), as part of general accounts of the toxicity of numerous metal salts to fish. Lloyd (1962) has reviewed the literature on some aspects of the toxicity of salts of the heavy metals. Jones (1964) has briefly reviewed most of the published work on zinc, as part of a general account of the effects of pollution on fish. Almost all the important studies concerning zinc have been published in the last five years. The present summary is based on my review of this subject (Skidmore, 1964), in the light of recently published work.

There have been numerous studies of the acute toxicity of zinc compounds to aquatic animals, and data from the more accessible references are summarized in Table 1.

Table 1. Survival of aquatic animals in water containing zinc.

| Original author | Source of data | Dilution water* | Temp. (°C) | Test Animals | Result*-** |
|---|---|---|---|---|---|
| Abbott, 1924 | Cairns & Scheier, 1957 | Devils Lake, Dakota | ? | small fish | dead after 8 hr. in Zn 15 |
| Affleck, 1952 | Table 5 | hatchery water, Ca 1.7, Mg 1.0 | 4-9 | rainbow trout eggs | 0% hatched in Zn 0.04 |
| " | Table 10 | " | 8-12 | rainbow trout fry | 46% survived 28 days in Zn 0.01 |
| " | " | " | " | " | 98% survived 28 days in Zn 0.003 |
| " | Table 11 | " | 9-12 | rainbow trout fingerlings | 0% survived 1 day in Zn 0.13 |
| " | " | " | " | brown trout fingerlings | 100% survived 20 days in Zn 0.13 |
| Anderson, 1944 | Table 1 | Lake Erie, Ca 31 | 25 | Daphnia magna | 16 hr. $LC_{50}$=Zn 19** |
| Anderson, 1948 | Table 1 | " | " | " | 64 hr. $LC_{50}$=Zn 0.072 |
| Cairns & Scheier, 1957 | Table 2 | synthetic water, Ca 11, Mg 4 | 18 | bluegills | 96 hr. $LC_{50}$=Zn 2.9-3.8 |

Table 1 (cont.)

| | | | | | |
|---|---|---|---|---|---|
| Cairns & Scheier, 1957 | Table 2 | synthetic water, Ca 11, Mg 4 | 30 | bluegills | 96 hr.$LC_{50}$=Zn 1.9-3.6 |
| " | " | synthetic, Ca 8700, Mg 3000 | 18 | " | 96 hr.$LC_{50}$=Zn 10.1-12.5 |
| " | " | " | 30 | " | 96 hr.$LC_{50}$=Zn 10.2-12.3 |
| Carpenter, 1927 | Table E | distilled water? | 18? | Phoxinus phoxinus (minnows) | mean survival time of 200 min. in Zn 330 |
| Ellis, 1937 | p. 430 | hard water | ? | goldfish | few survived 5 days in zinc sulphate 100 |
| Fowler, 1931 | Anderson, 1948 | well water | ? | Daphnia longispina | Zn 65 toxic in 15 hr. |
| Goodman, 1951 | Table 1 | hard water | ? | rainbow trout fingerlings | 24 hr. $LC_{50}$=Zn 2-6 |
| " | Table 3 | " | ? | " | 48 hr. $LC_{50}$=Zn 3-4 |
| Grindley, 1946 | Cairns & Scheier, 1957 | ? | ? | rainbow trout | survival time of 133 min. in Zn 25 |
| Hutchinson, 1933 | Anderson, 1948 | pond water? | ? | Daphnia magna and D. pulex | survival time < 5 days in Zn 0.65 |
| Jones, 1938 | Table 3 | tap water, Ca 1 | 14-17 | Gasterosteus aculeatus (sticklebacks) | 8.5 day $LC_{50}$=Zn 0.3 |

Table 1 (cont.)

| | | | | | |
|---|---|---|---|---|---|
| Jones, 1938 | Table 3 | tap water, Ca 1 | 14-17 | Gasterosteus aculeatus (sticklebacks) | 108 hr. $LC_{50}$=Zn 0.7 |
| " | " | " | " | " | 6 hr. $LC_{50}$=Zn 20 |
| " | " | " | " | " | 143 min. $LC_{50}$=Zn 200 |
| Jones, 1940a | Table 1 | distilled water | 15-18 | Polycelis (planarians) | 48 hr. $LC_{50}$=Zn 12 |
| Jones, 1940b | p. 378 | soft river water, Zn 0.7-1.2, Pb 0.05 | ? | - | fish absent from river |
| Lloyd, 1960 | Fig. 1 | diluted well water, Ca 4.5, Mg 0.19 | 17.5 | rainbow trout fingerlings | 48 hr. $LC_{50}$=Zn 0.6 |
| " | " | diluted well water, Ca 19, Mg 0.79 | " | " | 48 hr. $LC_{50}$=Zn 2 |
| " | " | well water, Ca 120, Mg 5 | " | " | 48 hr. $LC_{50}$=Zn 4 |
| Mathews, 1904 | Table 2 | distilled water | 19-26 | Fundulus eggs | no embryos developed in Zn 40 |
| " | " | " | " | " | 18% of embryos developed in Zn 27 |
| Naumann, 1934 | Table 2 | hard water | ? | Daphnia magna | all dead in Zn 0.01 in 10 days |

Table 1 (cont.)

| | | | | | |
|---|---|---|---|---|---|
| Naumann, 1934 | Table 2 | soft water | ? | Daphnia magna | most survived in Zn 0.1 |
| Oshima, 1931 | Doudoroff & Katz, 1953 | distilled water? | 20-22 | young eels | 50 hr. $LC_o$=Zn 0.065 |
| " | " | " | " | " | 20 hr. $LC_{50}$=Zn 6.5 |
| Rushton, 1949 | Doudoroff & Katz, 1953 | tap water | ? | young carp | survival time < 1 day in Zn 0.5 |
| Sprague, 1964a | Fig. 1 | tap water $CaCO_3$ 20 | 15 | Salmo salar | ∞ $LC_{50}$=Zn 0.6 |
| Sreenivasan & Raj, 1963 | p. 363 | soft water? | 28-30 | Cyprinus carpio | 48 hr. $LC_{50}$=zinc sulphate 10-12 |
| " | " | " | " | Danio sp. | 48 hr. $LC_{50}$=zinc sulphate 10 |
| " | " | " | 26-29 | Tilapia mossambica | 48 hr. $LC_{50}$=zinc sulphate 10-15 |
| Thomas, 1915 | Doudoroff & Katz, 1953 | sea water | ? | Fundulus (marine strain) | zinc sulphate 200 non-toxic |
| " | " | fresh water | ? | Fundulus (freshwater strain) | survival time 2 days in zinc sulphate 10 |

Table 1 (cont.)

| | | | | | | |
|---|---|---|---|---|---|---|
| Wurtz, 1962 | Table 2 | water, | $CaCO_3$ 20 | 13 | Young Physa heterotropha (snails) | 96 hr. $LC_{50}$=Zn 1.4 |
| " | " | " | $CaCO_3$ 100 | " | " | 96 hr. $LC_{50}$=Zn 0.43 |
| " | Table 3 | " | $CaCO_3$ 20 | " | Helisoma companulata (snails) | 96 hr. $LC_{50}$=Zn 3.0 |
| " | " | " | $CaCO_3$ 100 | " | " | 96 hr. $LC_{50}$=Zn 0.87 |

* All concentrations expressed in parts per million.

** '16 hr. $LC_{50}$' means 'the lethal concentration killing 50% of the test animals in 16 hours'.

Similarly, '50 hr. $LC_0$' means 'the highest concentration killing 0% of the test animals in 50 hours'.

Concentrations reported to be lethal vary from 330 p.p.m. zinc (Carpenter, 1927) to 0.01 p.p.m. (Affleck, 1952). Apart from the large volume of largely repetitive work indicated in Table 1, interest in the toxicity of zinc has proceeded along two main lines. On the one hand, investigators have studied how different factors modify the toxicity of zinc. On the other, they have attempted to understand the mode of toxic action of the metal. In consequence, this summary falls into two parts.

b) Factors influencing the toxicity of zinc compounds

The most important factor influencing whether a given concentration of poison will kill an aquatic animal is the duration of exposure. Toxicity is influenced by environmental factors such as temperature, pH, dissolved oxygen, and carbon dioxide. The toxicity of zinc compounds in particular is modified by compounds of other heavy metals and of the alkaline earths. The complexing of zinc ions with different radicals may further modify its toxicity. The resistance of aquatic animals to a given toxic environment also differs at both the species and the individual levels. It may vary with life history and with prior exposure to the poison or to other environmental factors.

Some of the variation in the lethal concentrations reported in the literature is due to factors in the bioassay conditions which do not normally apply in nature. Between different investigations, the ratio of biomass of test animals to quantity of available poison has varied widely, at any given concentration. Finally, different workers have selected different physiological criteria to mark the response of the animal that would have terminated in its death.

The importance of those factors influencing the toxicity of zinc compounds will now be discussed.

(i) Relation between concentration and survival time

The survival time of aquatic animals exposed to a poison is inversely related to the concentration of poison. There may or may not be a lethal threshold concentration, defined by Bliss (1940) as the highest concentration that would just fail to kill under prolonged (theoretically infinite) exposure. Similarly, there may or may not be a lethal threshold time to death, that is defined here as the lowest exposure time necessary to produce death under the highest attainable concentration. In practice, the death of an animal is rarely taken as the end point of a toxicity bioassay, but rather some earlier reaction (such as immobilization) that is known to precede death.

There have been four studies of the time-concentration relationship for zinc. The first was by Jones (1938), who exposed juvenile sticklebacks (Gasterosteus aculeatus) to zinc sulphate dissolved in extremely soft tap water (1 p.p.m. calcium) at 14 to 17°C. He calculated the arithmetic mean survival time (end point unspecified), and plotted time against concentration (Fig. 1). He then repeated this work with mature sticklebacks (Table 2).

For exposure times greater than two days, the resistance of the two sets of animals appears to be approximately similar. From Fig. 1, Jones estimated the lethal threshold concentration to be less than 0.3 p.p.m. zinc. The data in Table 2 indicate that the lethal threshold time was less than 109 minutes.

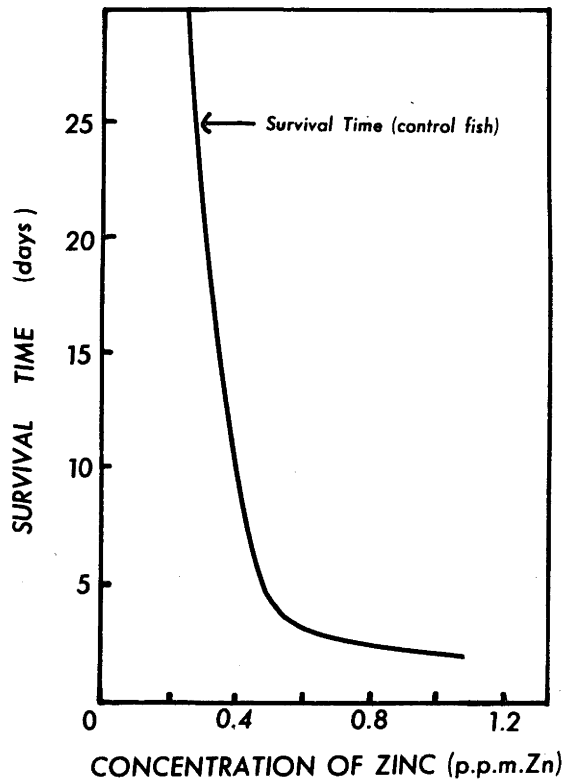
Anderson (1948) exposed 4-hour-old Daphnia magna to zinc chloride dissolved in soft water from Lake Erie, at a temperature of 25°C. For each group of daphnids exposed to the same concentration of zinc, he calculated the geometric mean time to immobilization, which may be defined by the following equation.

$$GMT_i = \sqrt[n]{T_1 \cdot T_2 \cdot T_3 \cdots T_n} \qquad (1)$$

where $T_1$, $T_2$, $T_3$,....$T_n$ are the individual times to

Fig. 1. Toxicity of zinc sulphate to juvenile sticklebacks (*Gasterosteus aculeatus*) in soft water, Ca 1 p.p.m. After Jones, 1938, Fig. 1.

Fig. 2. Toxicity of zinc chloride to *Daphnia magna* in Lake Erie water, Ca 31 p.p.m. After Anderson, 1948, Fig. 2.


Survival Time (control fish)
SURVIVAL TIME (days)
25
20
15
10
5
0
0.4
0.8
1.2
CONCENTRATION OF ZINC (p.p.m.Zn)

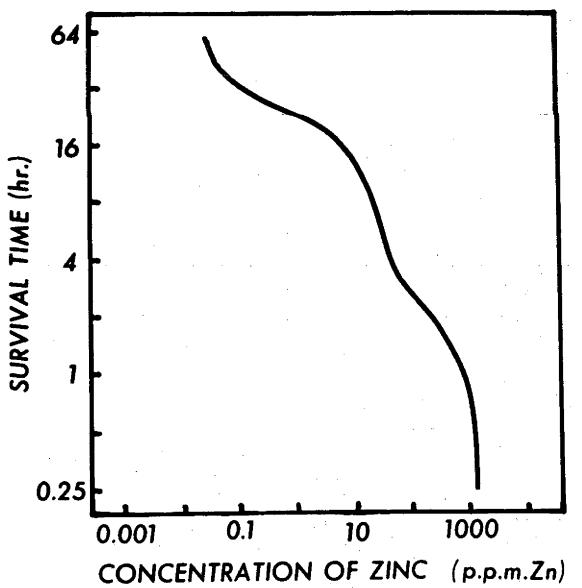

SURVIVAL TIME (hr.)
64
16
4
1
0.25
0.001
0.1
10
1000
CONCENTRATION OF ZINC (p.p.m.Zn)

Table 2. Toxicity of zinc sulphate to mature sticklebacks (*Gasterosteus aculeatus*) in soft water, Ca 1 p.p.m. Modified after Jones, 1938, Table 3.

| Concentration of zinc as Zn | Mean survival time |
|---|---|
| 300 p.p.m. | 109 min. |
| 100 p.p.m. | 207 min. |
| 30 p.p.m. | 5.3 hr. |
| 10 p.p.m. | 7.8 hr. |
| 3.0 p.p.m. | 16.5 hr. |
| 1.0 p.p.m. | 34 hr. |
| 0.3 p.p.m. | 8.5 days |
| 0.1 p.p.m. | 11.5 days |
| 0.0 p.p.m. | 10.5 days |

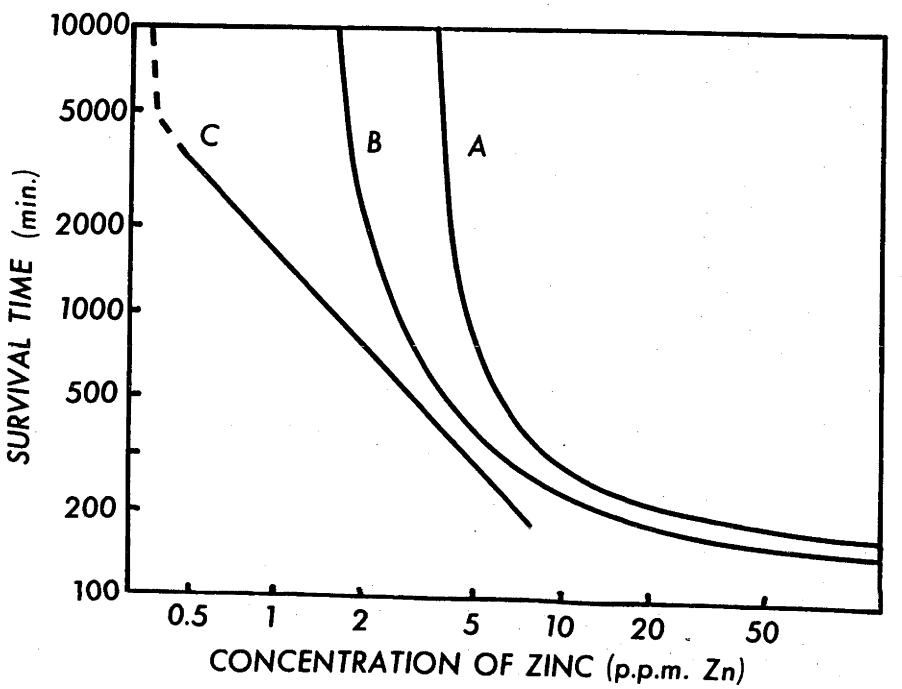
immobilization of n animals. Anderson then plotted mean time against concentration, both axes on a logarithmic scale (Fig. 2). He estimated that the lethal threshold concentration of zinc to Daphnia magna was less than 0.072 p.p.m.: the shape of the graph (Fig. 2) suggests that there is no threshold time of response. Anderson attributed the irregular shape of the regression line to the variable resistance of daphnids with age. This point will be discussed further in Section 2b (viii).

Lloyd (1960) exposed fingerling rainbow trout (Salmo gairdneri) to zinc sulphate dissolved in well water or diluted well water, at three levels of hardness, and at a temperature of 17.5°C. He graphically estimated the median time to immobilization of each batch of fish exposed to identical conditions by the method developed by Bliss (1937). Lloyd then plotted the logarithm of the median time against the logarithm of concentration of zinc, treating data from each hardness level separately (Fig. 3).

Curve C shows that the relationship between the logarithm of concentration of zinc and the logarithm of survival time was linear in soft water, within the range of concentrations examined. Regression lines of this type may be fitted to Ostwald's equation (1907),

$$C^n T = K \qquad (2)$$

Fig. 3. Toxicity of zinc sulphate to rainbow trout in waters of different total hardness. A, total hardness 320 p.p.m. as $CaCO_3$. B, 50 p.p.m. as $CaCO_3$. C, 12 p.p.m. as $CaCO_3$. After Lloyd, 1960, Fig. 1.


10000
5000
2000
1000
500
200
100
SURVIVAL TIME (min.)
C
B
A
0.5
1
2
5
10
20
50
CONCENTRATION OF ZINC (p.p.m. Zn)

where C = concentration, T = time, and n and K are constants. Lloyd's values for n and K were 1.06 and 1650 minutes respectively. As zinc is known to be an essential element in animal metabolism (e.g. Vallee, 1959), it clearly cannot be toxic in minute traces. It follows that a certain lethal threshold concentration is necessary to kill an aquatic animal, and that Curve C cannot therefore extend as far as the Y axis. A theoretically possible continuation of Curve C is shown by means of a dotted line. On the basis of further experimental work, Lloyd (1961b, Fig. 2a) decided that the regression line was in fact curvilinear, with a threshold concentration of 0.56 p.p.m. zinc.

The data for hard water indicate a curvilinear relationship between the logarithms of concentration and time (Curves A and B). Lloyd subtracted appropriate constants from concentration and time ($C_S$ and $T_S$ respectively), until the relationship between the logarithms of $(C - C_S)$ and $(T - T_S)$ was linear. The equation of the resulting regression line was developed by Wuhrmann (1952).

$$(C - C_S)^n (T - T_S) = K \qquad (3)$$

Lloyd's values for the four constants $C_S$, $T_S$, n and K, to fit his data, were:-

| | Curve A | Curve B |
|---|---|---|
| $C_S$ | 3.5 p.p.m. Zn | 1.5 p.p.m. Zn |
| $T_S$ | 160 minutes | 140 minutes |
| n | 1.24 | 1.43 |
| K | 813 minutes | 933 minutes |

Both Lloyd and Wuhrmann assumed that the mathematical constants $C_S$ and $T_S$ indicated lethal threshold concentration and lethal threshold time, respectively.

Sprague (1964a) repeated Lloyd's (1960) work using Atlantic salmon, Salmo salar. Subsamples of five salmon parr were exposed to a series of concentrations of zinc sulphate, at a hardness of 20 p.p.m. $CaCO_3$, temperature of 15°C., and pH between 7.1 and 7.5. The relation between log. concentration of zinc and log. median survival time (graphically estimated) was linear, down to a sharply defined lethal threshold concentration of 0.6 p.p.m. zinc (Sprague, 1964a, Fig.1). The data may be fitted to Equation 2, the values of n and K being .90 and 18 hours, respectively.

In all the data presented so far, the zinc was probably in the form of zinc ions ($Zn^{++}$). To test whether suspended zinc (possibly $ZnCO_3$) was also toxic, Lloyd (1960) exposed batches of trout to three treatments. In the first, there was no zinc; in the second, 9 p.p.m. suspended zinc, and in

the third, 18.5 p.p.m. There were approximately 11 p.p.m. zinc in solution in each case. Survival time was 285 minutes in the first treatment, 180 minutes in the second and only 162 minutes in the third. The suspended zinc thus reduced survival time substantially. Sprague (1964a) in contrast, found that up to 3 p.p.m. suspended zinc was non-toxic at pH 9.

The toxicity of soluble complex compounds containing zinc has been considered only by Doudoroff (1956). He found that the toxicity of the zinc cyanide ion ($Zn(CN)_4^{--}$) to Pimephales promelas (a minnow) was greater than could be predicted from that of the zinc ion and the cyanide ion taken separately. For example, half the fish were killed in 96 hours by either 0.23 p.p.m. cyanide (as the sodium salt) or 0.18 p.p.m. cyanide plus 0.125 p.p.m. zinc. Doudoroff's results therefore suggest synergism, as defined in Section 2b (iii).

The toxicity of complex compounds involving heavy metals apparently shows no consistent pattern. Doudoroff (1956) estimated the toxicity of cadmium cyanide to be greater, and the toxicities of copper and nickel cyanides to be less, than would be predicted from the toxicities of the appropriate ions taken separately. The only other comparable study has been by Jones (1940c), who concluded that the double chlorides of mercury and sodium were less toxic than would be

expected from the toxicities of mercury, sodium and chloride ions taken separately.

(ii) Effects of salts of the alkaline - earth metals

Lloyd (1960) considered hardness to be the most important single factor modifying the toxicity of zinc ions. He measured the survival time of rainbow trout in a series of concentrations of zinc, at three hardness levels. His methods have been described in Section 2b (i) and his results are presented in Fig. 3. The hardness of the three dilution waters was made up as follows:-

| Curve | Total hardness (p.p.m. $CaCO_3$) | Total calcium (p.p.m. Ca) | Total magnesium (p.p.m. Mg) |
|---|---|---|---|
| A | 320 | 120 | 5 |
| B | 50 | 19 | 0.79 |
| C | 12 | 4.5 | 0.19 |

In each case, 94% of the hardness was due to calcium ions.

Lloyd observed that the effect of hardness increased with increase in period of survival, until there was a ten-fold difference between the toxicities of zinc in the hardest and softest water over 2.5 days' exposure. As the curves were of different shapes, Lloyd deduced that the ratio between the toxicities of zinc at any two hardness levels was not consistant. However, he has recently modified this

opinion, and in the light of new evidence Lloyd and Herbert (1962) have postulated a linear relationship between log. total hardness and log. lethal threshold concentrations of zinc, copper and lead, respectively.

The remaining data on the toxicity of zinc in hard and soft water are of little more than historical interest. Jones (1938) observed that sticklebacks survived for 10 days in water containing 2 p.p.m. zinc and 50 p.p.m. calcium, but died in 2 p.p.m. zinc and no calcium. Cairns and Scheier (1957) estimated that the concentration of zinc killing half a sample of bluegills, (Lepomis macrochirus) in 4 days was 11.3 p.p.m. when the hardness was approximately 30,000 p.p.m. calcium carbonate, but only 2.8 when the hardness was approximately 40 p.p.m. calcium carbonate. Only Naumann (1934) has reported zinc to be less toxic in soft water than in hard water: his data are summarized in Table 1.

It has been known since the work of Ringer (1897) that the toxicity of some metal cations to aquatic organisms is reduced in the presence of other metal cations, notably calcium. This phenomenon is known as antagonism, and the literature on it is extensive. The more important reviews are by Heilbrunn (1937), Jones (1939b, 1964), and Doudoroff and Katz (1953).

Except in the study by Naumann (1934), antagonism has been detected in every case where the toxicity of heavy metal ions has been observed in the presence of ions of the alkaline earths. By far the most important contribution on antagonism involving the heavy metals has been by Jones (1939a), who studied the toxicity of copper nitrate to tadpoles of Bufo bufo, in the presence of the nitrates of either calcium, magnesium, strontium, or barium. At the two levels of copper investigated, Jones found that tadpoles survived longest when the concentration of the alkaline earths was approximately 0.1 normal. With 320 p.p.m. copper present, strontium caused the greatest antagonism, followed by calcium, magnesium, and barium, in that order. With 64 p.p.m. copper present, calcium exerted a greater influence than strontium, followed as before by magnesium and barium. Antagonism was also demonstrated between strontium and nickel ions, in a range of concentrations of both. In contrast, the toxicities of lead nitrate and cadmium nitrate were not reduced by salts of the alkaline earths.

A major criticism of Jones' work is that he only studied the effects of highly toxic solutions of heavy-metal ions, although he himself stated (Jones, 1939a) that the mode of toxic action of the heavy metals undoubtedly varies with concentration. This deficiency has been to some extent filled by the work of Lloyd (1960), who recorded in effect the

antagonism of three concentrations of calcium to concentrations of zinc ranging from 0.5 to 50 p.p.m. Unfortunately, no author since Jones has recompared the antagonistic effects of the four alkaline-earth metals, or has remeasured at what concentration these effects reach a maximum.

Two hypotheses have been proposed concerning the mechanism of antagonism by the alkaline-earth metals. The relevant literature has been briefly reviewed by Jones (1939b), who attributed the first hypothesis to work by Loeb and Osterhout (Osterhout, 1922), and the second to Heilbrunn (1928, 1937).

According to the first hypothesis, one compound antagonizes another by reducing the permeability of cell membranes, thereby reducing the rate of transport of the second compound into the tissues. Loeb and Osterhout demonstrated that the permeability of sodium and potassium ions into tissues was reduced by the antagonism of ions of the alkaline earths and heavy metals. The antagonism of the alkaline earths to toxic solutions of zinc may also be owing to a reduction in the permeability of cell membranes (Department of Scientific and Industrial Research, 1958, Figs. 32 & 33). Trout reared in hard water, but exposed to a toxic concentration of zinc in soft water, survived as long as trout reared in either hard or soft water and exposed to zinc in hard water. In all three cases, survival time was approximately the same, and

much longer than the survival time of trout reared in soft water and exposed to zinc in soft water. Lloyd (1962), commenting on the above data, suggested that the protection afforded by the alkaline-earth metals was internal, and cited in support the observation by Houston (1959) that trout reared in hard water contain more calcium. However, as the fish reared in soft water and tested in hard water had no time in which to absorb calcium or magnesium ions, prior to their exposure to zinc, it is suggested here that the antagonism of the zinc ions by the hard water acted at the surface of the fish. This question could perhaps be settled using a convenient radioisotope, such as calcium-45.

The second hypothesis is that most metal ions poison organisms by their ability to coagulate the contents of cells, but that coagulation was inhibited by calcium ions. To date, no-one has tested this theory histologically, but it has been observed that fish mucus became coagulated by zinc or lead ions, both on the skin (Carpenter, 1927; Jones, 1938) and in vitro (Jones, 1938). In both cases, coagulation was inhibited if sufficient calcium ions were present. On the other hand, there is slight evidence that salts of the heavy metals are not general internal poisons to fish. Schweiger (1957) injected substantial amounts of either manganese or cadmium salts into the body cavity and alimentary canal of carp, without apparent damage.

It is clear that the mechanism of antagonism, concerning both zinc and other heavy metals, is poorly understood. The two hypotheses cited above do not appear to be mutually exclusive, and further evidence is needed to evaluate them.

(iii) Effect of salts of the heavy metals

If an aquatic animal is exposed simultaneously to two poisons, the result may either be predictable from the toxicity of each poison taken separately (additive effect), be greater than predicted (synergism), or be less than predicted (antagonism). Under different conditions, mixtures of zinc ions and the ions of other heavy metals have been shown to demonstrate the first two effects, which will be illustrated here. Antagonism has already been discussed in Section 2b (ii).

The studies of Bandt (1946) and Doudoroff (1952) have been reviewed by Doudoroff and Katz (1953). Bandt exposed trout and roach to mixtures of sulphates of the heavy metals in soft tap water. His results were:

| | |
|---|---|
| zinc + cadmium | additive effect |
| zinc + nickel | synergistic effect |
| zinc + copper | strongly synergistic, up to five times more toxic. |

Doudoroff (1952) exposed minnows (Pimephales), for 8 hours, to solutions containing ions of zinc or copper or a mixture of the two, in soft water. Those fish in either 8 p.p.m. zinc or 0.2 p.p.m. copper survived: others in 1 p.p.m. zinc plus 0.025 p.p.m. copper died. Thus Doudoroff and Bandt both observed pronounced synergism between zinc and copper in soft water.

Lloyd (1961b) exposed rainbow trout to solutions of zinc sulphate, copper sulphate, and a mixture of the two, made up in hard water (320 p.p.m. $CaCO_3$) and soft water (15-20 p.p.m. $CaCO_3$). The mixture was always in the ratio of six parts of zinc to one of copper, by weight. About six concentrations of each of the six series of treatments were tested. The experiment in soft water was terminated after 7 days. The concentration of zinc that immobilized half the trout in that time was 10.5 times the concentration of copper. Lloyd assumed that the same ratio held over all exposure times less than 7 days, and fitted by eye the best curve of survival time upon concentration of poison that satisfied both the zinc and the copper data. He then fitted the data for the zinc-copper mixture about the same curve. Data falling below the line indicated synergism, because survival time was lower than predicted. This occurred where the concentration of zinc exceeded about 1.8 p.p.m. and copper 0.3 p.p.m. The

effects of the zinc and copper at lower concentrations were additive. Lloyd treated the data from the experiments using hard water by the same method and found the effects of zinc and copper were additive at all concentrations, but the most toxic mixture tested only contained about 6 p.p.m. zinc plus 1 p.p.m. copper.

Sprague (1964a) found that the survival times in soft water of Atlantic salmon exposed to two concentrations of an equitoxic mixture of zinc and copper were about half the estimated times, indicating pronounced synergism. The lower concentration tested was about 1.5 times the lethal threshold concentration.

(iv) Effect of dissolved oxygen

Lloyd (1960) exposed rainbow trout to five lethal concentrations of zinc sulphate at three non-lethal concentrations of dissolved oxygen each, in hard water ($CaCO_3$ 320 p.p.m.), using methods already described in Section 2b (i). He calculated that, over an exposure period of 1000 minutes, the concentration of zinc necessary to kill half the fish was 1.4 times greater at an oxygen concentration of 8.9 p.p.m. than it was at 3.8 p.p.m.

Westfall (1945) found that lead was more toxic to goldfish in water low in oxygen, and Wuhrmann (1952) observed

that ammonia, cyanides and phenols have all been reported to be more toxic to fish in water low in oxygen. Lloyd (1961a) considered the effect of oxygen concentration on the toxicity to rainbow trout of poisons in general. He illustrated his discussion with examples from his own work on zinc sulphate (described above), lead nitrate, copper sulphate, and a mixture of phenols.

Lloyd (1961a) measured the increase in toxicity of a poison which occurs when the dissolved oxygen concentration is reduced, by dividing the lethal threshold concentration obtained under air-saturation conditions ($X_S$) by the lethal concentration obtained for a lower oxygen concentration (X). Different values for $\frac{X_S}{X}$ were then plotted against the corresponding concentrations of oxygen, expressed as percent air-saturation. For all four poisons, the value of $\frac{X_S}{X}$ increased from 1.0 at 100% air-saturation, to 1.4 at 40% air-saturation. In further work on the toxicity of ammonium chloride, Lloyd found that $\frac{X_S}{X}$ increased to 2.2 at 40% air-saturation, based on an exposure time of 500 minutes. He was able to demonstrate that this high value of $\frac{X_S}{X}$ was caused by an increase in pH at the gill epithelium, which was caused by a reduction in concentration of excreted carbon dioxide, which was in turn associated with a drop in oxygen consumption.

As $\frac{X_s}{X}$ is similar for different poisons (under otherwise identical conditions), Lloyd suggested that low oxygen concentration causes a physiological response independent of the effect of the poison, the most obvious reaction being an increase in ventilation rate, that is to say, in the rate of flow of water over the gills. This in turn would increase the amount of poison reaching the gill epithelium, which Lloyd assumed to be the site where most poisons are absorbed by fish. He cited the work of Weiss and Botts (1957) as showing that the toxicity of an organic poison to several species of fish was increased by either an increase in oxygen uptake or by low oxygen concentration. In both cases, Weiss and Botts thought that the toxicity of the poison was proportional to the rate of respiratory flow. Lloyd then argued, on theoretical grounds, that this conclusion was justified.

If Lloyd is correct in concluding that the survival time of a fish in a toxic solution is proportional to the rate of flow of water over its gills, then it follows that any environmental change that causes a change in ventilation rate will have the same effect as change in oxygen tension. Lloyd based his hypothesis on a comparison of concentrations of five poisons under only three levels of dissolved oxygen, and under only one level each of temperature, water hardness and exposure time. The importance of the theory would justify a much

more extensive study of a number of the variables influencing toxicity.

(v) Effect of activity

Herbert and Shurben (1963) have demonstrated that enforced activity increased the susceptibility of rainbow trout to zinc poisoning. This increase in susceptibility was not very great, and at swimming speeds close to the maximum which trout can sustain for 2 days, the concentration of zinc sulphate killing half the fish in 48 hours (48 hr. $LC_{50}$) was only 0.7 of the value obtained in still water. The increase in toxicity of the zinc was related to an increase in oxygen uptake, and could be accounted for by an increase in ventilation rate. A similar result was obtained by the same authors using ammonium chloride as the toxic agent. This work gives some support to the hypothesis of Lloyd (1961a) which was discussed in the previous section.

(vi) Effect of carbon dioxide

There is no direct evidence about the effect of carbon dioxide concentration on the toxicity of zinc, and only scanty indirect data. Lloyd (1960) compared the survival times of rainbow trout in five concentrations of zinc, made up in two batches of hard water (hardness probably equivalent

to 320 p.p.m. $CaCO_3$). The hardness in the first batch was mainly due to calcium bicarbonate, and in the second mainly to calcium chloride. Survival times in the bicarbonate solutions were consistently lower. Possibly this difference was due to the toxicity of either bicarbonate ions or carbon dioxide, or a combination of the two, but the relative concentrations of each cannot be deduced from Lloyd's data.

(vii) Effect of temperature

There have been three studies of the effect of temperature on zinc toxicity. Lloyd (1960) compared the survival times of rainbow trout in four concentrations of zinc, in hard water, tested at four temperature levels. Fish were held for 5 days and then tested at temperatures of 13.5, 15.5, 18.5, or 21.5°C. Survival times were generally lower in the warmer water, but the threshold concentration appears to have been unchanged. Lloyd calculated that a rise in temperature from 12 to 22°C. reduced the survival time in relatively high concentrations of zinc by a factor of 2.35. Cairns and Scheier (1957), in apparent contrast to Lloyd, observed little difference between the toxicity of zinc to bluegills, at either 18 or 30°C., over long exposure periods. On the other hand, Sprague (1964a) found that Atlantic salmon survived four times longer in 3 p.p.m. zinc at 5 than at

15°C., and the lethal threshold concentration was apparently raised 1.5 times by a similar decrease in temperature.

(viii) Resistance of aquatic animals

Data from Table 1 indicate that widely different concentrations of zinc compounds have been reported as toxic to different species of aquatic animals. It is not possible however to list the different species in order of their resistance to zinc, because bioassay conditions have differed greatly. No author has effectively compared the relative resistance of any two species of aquatic animals to zinc poisoning. This information cannot be deduced from the known resistance of aquatic animals to other poisons because a species that is more resistant than another to one poison may be less resistant to a second poison (e.g. Wuhrmann, 1952; Applegate et al, 1957).

Resistance to poisons also varies greatly between individuals of the same species. Lloyd (1960) found that the logarithms of individual survival times of trout in a given concentration of zinc were normally distributed. Because of this, he was able to estimate the median results for his data graphically, by plotting the logarithms of survival time against probit kill, as indicated in Section 2b (i). Weiss and Botts (1957) found that larger fish tended to be more

resistant to an organic poison than smaller fish of the same species, and that their resistance appeared to be related to their oxygen consumption.

The resistance of a population of animals to a poison may vary as a result of at least three factors: acclimatization (or adaptation) to the poison or some other environmental factor, development of a new phase in life history, and survival of a resistant group by selective mortality. Several authors have discussed one or more of these factors with reference to zinc. Each factor will now be considered in turn, beginning with data indicating acclimatization.

Lloyd (1960) exposed two batches of rainbow trout to 3.5 p.p.m. and 2.5 p.p.m. zinc respectively, in hard water, for 14 days. At the end of this period, the fish were transferred to 10 p.p.m. zinc, together with a control group which had previously been held in tap water. The fish previously held in 3.5 p.p.m. zinc survived 500 minutes, those held in 2.5 p.p.m. survived 400 minutes, and the control group only 290 minutes. In another experiment, Lloyd held three batches of trout in 9.45, 6.3, and 3.65 p.p.m. of dissolved oxygen for 18 hours. He then exposed subsamples of each batch to five lethal concentrations of zinc in hard water. Fish held in water low in oxygen, and exposed to zinc at the same oxygen tension survived longer than fish not acclimatized to low

oxygen tension. Finally, it has already been mentioned (Section 2b (ii)) that trout held by Lloyd in hard water, and then exposed to a lethal concentration of zinc in soft water, survived longer than trout held and tested in soft water (D.S.I.R., 1958). Lloyd was thus able to demonstrate that the resistance of rainbow trout to zinc poisoning was raised if the fish became acclimatized to either a sublethal concentration of zinc, to a low concentration of dissolved oxygen, or to hard water. The resistance of fish to several organic poisons has also been shown to increase following acclimatization to the test conditions (Sumner & Wells, 1935; Weiss & Botts, 1957). In both studies, resistance was apparently related to oxygen uptake.

Observations on the resistance to zinc poisoning of test animals of different ages have been noted by Jones (1938) and Anderson (1948). Jones' study of the toxicity of zinc sulphate to sticklebacks has already been summarized in Section 2b (i). Jones found that the resistance of juveniles (length 18-20 mm.) and sexually mature adults (length 45-50 mm.) was approximately similar throughout the range of concentrations of zinc tested. Anderson's work on the toxicity of zinc chloride to daphnids is also discussed in Section 2b (i). Anderson attributed the irregular shape of the regression line in Fig. 2 to variations in the resistance of the test

animals. He cited an earlier study (Anderson & Jenkins, 1942) showing that at 25°C. Daphnia magna moults at about 20 hours after release from its mother. A reduction in slope of the regression line at about this time indicates a reduction in resistance of the test animal. Anderson suggested that Daphnia magna is less resistant to zinc poisoning at ecdysis because penetration of the animal by zinc ions is then easier.

An increase in resistance of a population of aquatic animals to any poison by selective mortality alone has not yet been reported, either in the laboratory or the field, although the phenomenon is well known among insects exposed to insecticides and micro-organisms exposed to antibiotics (e.g. Brown, 1960; Miller & Bohnhoff, 1950). However, Paul (1952) has described an interesting situation in California, where the concentration of copper in certain polluted streams was high enough to kill introduced, hatchery-reared fish, although the resident fish population was apparently unharmed. Paul attributed the resistance of the native fish to acclimatization, but from the meagre data, selective mortality seems equally possible.

The only other reports indicating variable resistance to zinc poisoning are by Goodman (1951) and Affleck (1952). Goodman maintained fry of rainbow trout in tap water containing 1 p.p.m. zinc. Samples of twenty fry of known ages were

then exposed to a range of concentrations of zinc for 2 days. A selection of Goodman's results is given in Table 3.

Goodman concluded that the resistance of trout fry to zinc increased with age. Affleck found that trout fry previously held in water contaminated with an unspecified concentration of zinc survived a subsequent exposure to a low concentration of zinc better than fry of the same age reared in zinc-free water. Affleck attributed this increased resistance to acclimatization.

In both studies, the mortality of trout fry during the rearing period was unspecified. Therefore, in either case, the observed differences in resistance may have been due to selective mortality of the fry during rearing caused by low concentrations of zinc, or to increasing acclimatization. With Goodman's data, there is the third possibility that resistance may have increased with age. Because of this uncertainty, the conclusions of both authors must be treated with caution.

(ix) Other factors

The remaining factors that may modify the reported toxicity of zinc result from limitations of the toxicity bioassay The number and size of animals in the test solutions will be considered first.

Table 3. Survival of samples of 20 rainbow trout (Salmo gairdneri) following exposure to solutions of zinc sulphate for 2 days. Data from Goodman, 1951.

| Age of fish | Concentration of zinc as Zn | Number of survivors |
|---|---|---|
| 2 weeks | 3 p.p.m. | 11 |
| 2 weeks | 4 p.p.m. | 2 |
| 4 weeks | 4 p.p.m. | 2 |
| 8 weeks | 4 p.p.m. | 18 |
| 10 weeks | 4 p.p.m. | 20 |

The absorption or precipitation of dissolved zinc by aquatic animals has been demonstrated by Jones (1938), Saiki and Mori (1955), Joyner (1961), and several other workers. It follows that when animals are exposed to zinc in a limited volume of solution, the concentration of zinc will be reduced by the animals. If the biomass of the animals is large, and the volume of solution is small, the concentration of zinc may be substantially reduced, and the survival time of the animals in a lethal concentration will then be unnaturally high. In a toxicity bioassay, the importance of minimizing the ratio of biomass of test animals to mass of available poison is thus apparent.

The effect of high ratios of mass of animals to mass of poison has been demonstrated by Carpenter (1927, 1930) in her work on the toxicity of lead nitrate to minnows. She found that when a small sample of solution was assayed several times using a series of fresh fish, each fish survived longer than the previous one. In another series of experiments, survival of fish in small samples of toxic solution was compared with the survival of similar fish in larger samples of the same concentrations. The smallest concentration of lead that produced the maximum toxic effect was 6200 p.p.m., using 1.8 fish in 500 ml. solution (Carpenter, 1927, p. 383). From these figures it can be calculated that the critical ratio of

biomass of fish to mass of available lead was 0.6:1. To maintain this ratio using a solution of 1 p.p.m. lead, and one fish weighing 1 gram, a volume of not less than 1700 litres would be necessary! Satisfactory ratios may be achieved more conveniently by using very small test animals, or by continually replacing the solution.

The critical ratio of test animals to available zinc has not been determined. Most of the published work on the toxicity of zinc does not include the weight of the test animals, so that the ratios cannot usually be calculated. Jones (1938), Goodman (1951), Cairns and Scheier (1957), and Lloyd (1960) all appear to have used fish:: zinc ratios of at least 500:1. A notable exception is Anderson (1948), who achieved a ratio of about 10:1.

Another factor that may partly explain the wide range of zinc concentrations reported as lethal, is the choice of end point. Different investigators have selected a variety of responses to mark the reaction of test animals to a toxic environment. The subject has been discussed by Wuhrmann (1952), who listed the following possible end points to a toxicity bioassay:

1. Initial response to toxic action (such as increased ventilation rate);
2. Manifestation time, or overturn time;

3. Lethal time, or the onset of irreversible change;

4. Death.

Of the four, Wuhrmann considered overturn time to be the most precise and the easiest to ascertain. However, most investigators of zinc toxicity have chosen some manifestation of death as the end point. Carpenter (1927) selected total immobilization, and Anderson (1948) the cessation of swimming. Cairns and Scheier (1957) and Lloyd (1960) established immobilization of the gills as the end point. Most other workers terminated the bioassays at death, without defining how this was determined.

Finally, an irritating amount of ambiguity in the literature has arisen from authors' not stating the chemical formulas on which their lethal concentrations were based, and from doubts about the purity of their compounds. Doudoroff and Katz (1953, pp. 817-18) have discussed these objections.

c) Toxic action of zinc salts on aquatic animals

The factors known to influence the toxicity of zinc salts to aquatic animals have been enumerated. The toxic action of zinc will now be considered, beginning with a survey of morphological and physiological changes caused by zinc. The entry into, and accumulation of zinc in the body will then be

discussed. Finally, theories concerning the toxic action of zinc will be evaluated.

(i) Changes in morphology

It is convenient to describe separately the effects of acutely toxic and chronically toxic concentrations of zinc, because exposure to different concentrations produces different results. Early observations on morphological changes in aquatic animals were limited to changes caused by acutely toxic concentrations, and this work will be considered first.

Carpenter (1927) and Jones (1938) reported gill damage and copious secretions of mucus in minnows (Phoxinus phoxinus) and sticklebacks (Gasterosteus aculeatus) killed by high concentrations of zinc. The mucus was believed to be the major cause of death, through mechanical obstruction of the gills. Carpenter showed that the mucus probably contained zinc because it turned brown when treated with ammonium sulphide. Jones demonstrated that a toxic solution of zinc sulphate precipitated filtered eel mucus, but neither the clogging of the gills nor cellular damage were apparently confirmed histologically by either author, and it is believed that this may be a crucial shortcoming of their evaluations.

Lloyd (1960) briefly reported the results of a histological study made for him by Dr Gwyneth Parry, on the gills of

trout which had been exposed to different concentrations of zinc. In 20 p.p.m. zinc, cytological breakdown of the gill epithelium occurred within 2.5 hours, but it is not clear whether or not the fish had overturned. In 4 p.p.m. zinc, the gill lamellae became swollen before death. No change was detected in fish exposed to 3 p.p.m. zinc for 2 days.

Some additional information about Lloyd and Parry's work is supplied by the Department of Scientific and Industrial Research (1960, p. 83). Unspecified but toxic concentrations of zinc, lead, and copper salts all acted on the gills of trout as follows. After half the expected survival time had elapsed, the epithelium began to separate from the filaments and lamellae. By the time the fish had overturned, approximately half the epithelium had been sloughed off, and at death, three-quarters. Thus at death, three-quarters of the gills apparently consisted of an undamaged net work of blood vessels and connective tissue from which the gill epithelium had been removed. It is not stated whether the sloughed-off cells clogged the intact gill tissue, or whether they became detached.

Lloyd (1960) reported further that Parry rarely observed any precipitated mucus in the gill chamber at any concentration of zinc, but no special staining technique for mucus (e.g. the periodic acid - Schiff reaction) appears to have been

employed. Using a radioisotope of zinc ($Zn^{65}$), Lloyd detected zinc in mucus secreted over the body surface of zinc-immobilized fish, but he did not see any coagulated mucus on the gills. On the basis of his and Parry's work, Lloyd considered that mucus did not clog the gills of fish that were killed by zinc.

The only published information on morphological changes in aquatic animals caused by chronically toxic concentrations of zinc salts is contained in two careful studies by Crandall and Goodnight (1962, 1963). These authors (1962) exposed seventy-nine new-born guppies (Lebistes reticulatus) to 1.15 p.p.m. zinc, in tap water (hardness 80 p.p.m. $CaCO_3$) at 25 to 27°C. The ratio of fish to available zinc was not stated, but was probably in the order of 20,000:1. It is possible therefore that the fish were exposed to much lower concentrations of zinc than the authors believed. It is significant that Cairns and Scheier (1957) found that bluegills (Lepomis macrochirus) exposed to 1 p.p.m. zinc were able to precipitate most of it as mucus within 24 hours.

Crandall and Goodnight found that the guppies reared in zinc solution grew less rapidly than fifty-four similar fish in zinc-free water. The experimental group had a higher mortality rate and showed less sexual dimorphism. For example, after 90 days the median weights of the experimental

and control groups were 23 and 52 mg. respectively. Similarly, the cumulative mortality was 41% compared with 9%. Only one of the experimental fish developed a gonopodium (male reproductive organ) compared with 30 to 40% of the control fish. In a further experiment, the mortality of twenty-two guppies exposed to 2.3 p.p.m. zinc was 60% in from 49 to 69 days, at which point the study was terminated.

In their second study (1963), Crandall and Goodnight again exposed new-born guppies to either 1.15 or 2.3 p.p.m. zinc, under the same conditions as before. Live fish were removed periodically, preserved, sectioned longitudinally, stained with haematoxylin and eosin, and examined histologically. After 55 to 65 days in 1.15 p.p.m. zinc, blood vessels in the liver were poorly developed, the mesenteries were practically devoid of fat, the kidney tubules and glomeruli were distended, the lymphoid tissue in the kidneys was reduced, and the gonads were under-developed. After 95 days, the liver contained large vacuoles, and granulocytes had accumulated in the heart muscle. The kidney tubules were even more expanded, the spleen was under-developed and only one quarter of the fish were sexually mature. Control fish of the same age were all sexually mature and showed no abnormalities.

After 58 to 70 days in 2.3 p.p.m. zinc, the liver had degenerated and contained large vacuoles and irregular nuclei. The mesenteries contained no fat, the pancreas was undersized, the kidneys were distorted and haemorrhaged, and the skeletal muscles were under-developed and vacuolated. In none of the fish examined was there any gill damage.

The available information indicates that rapidly-lethal concentrations of zinc definitely cause severe cytological damage to the gills and some coagulation of mucus over large areas of the body. Opinion is divided as to whether coagulation in the gill cavity is severe enough to clog the gills. No other morphological changes have been observed. Chronically toxic concentrations of zinc, on the other hand, cause no damage to the gills, but induce extensive deterioration to liver, kidneys, hearts, skeletal muscle, gonads and spleen.

Studies by several authors concerning the effects of different poisons on a variety of fish indicate that none of the morphological changes just described are peculiar to zinc-poisoning. Gill damage, sometimes accompanied by coagulated mucus on the gills, has been reported in fish exposed to rapidly lethal concentrations of lead salts (Carpenter, 1927; Ellis, 1937; Jones, 1938, 1939c), copper salts (Department of Scientific and Industrial Research, 1960), salts of various other heavy metals (Schweiger, 1957),

trifluoromethyl nitrophenol (Christie & Battle, 1963), and dodecylbenzene sulphonate (Schmid & Mann, 1961, 1962). Observations of gill damage, in the last four papers mentioned, were supported by histological data. The significance of gill damage caused by fish-poisons will be discussed in Section 2c (v).

(ii) Changes in physiology and behaviour

Jones (1938) observed that the rate of opercular movements of three-spined sticklebacks (Gasterosteus aculeatus) increased when the fish were exposed to an acutely toxic concentration of zinc sulphate. In tap water, the opercular rate was approximately 100 beats per minute. When the fish were introduced into a toxic solution of zinc sulphate (either 2 p.p.m. or 10 p.p.m. zinc) the rate increased steadily to 240 beats per minute and then declined rapidly until death. Naturally, the response of fish in the higher concentration was quicker. Fish removed from the toxic solutions, while the opercular rate was 240 per minute, recovered, and the rate returned to normal. Increases in opercular rates have also been recorded in fish exposed to acutely toxic concentrations of lead nitrate, copper sulphate and mercuric chloride (Jones, 1938; Carpenter, 1927).

In a later study, Jones (1947a) was able to demonstrate that an increase in opercular rate coincided with a decrease in oxygen consumption when sticklebacks were exposed to lethal concentrations of lead, copper, and mercuric salts. Carpenter (1927) made a parallel observation that carbon dioxide production decreased as opercular rate increased when sticklebacks were exposed to a toxic solution of lead nitrate. Thus in both studies, the rate of gill movements increased as the rate of gas transfer decreased. However, when Jones exposed sticklebacks to toxic solutions of cyanide and sulphide, oxygen consumption and opercular rate both decreased.

Jones explained this difference in response as follows. Cyanides and sulphides inhibit tissue respiration. The carbon dioxide concentration of the blood is therefore lowered, causing both gill movements and gas exchange to decline. Heavy-metal salts, in contrast, do not significantly inhibit tissue respiration. As gas exchange becomes increasingly prevented at the gill surface, so the carbon dioxide content of the blood rises. The respiratory centre is then stimulated, causing the opercular movements to increase in rate and amplitude.

It follows from Jones' argument that the drop in oxygen consumption on exposure to heavy-metal salts is probably not

caused by a reduced flow of water through the gills but by the reduced efficiency of the gills (owing perhaps to gill damage, circulatory changes or coagulated mucus). Unfortunately, no information is available on any of these three points, with the exception of an interesting observation by Ellis (1937).

Ellis studied the rate of heart beat of the carp, by inserting a needle into the pericardial cavity until the point just touched the ventricle of the heart. As the heart continued to beat, so the visible end of the needle osscilated. Ellis observed that when a carp was exposed to a solution of the salt of a heavy metal, the heart beat remained strong while the gills apparently became clogged with mucus - and presumably damaged cytologically. At some critical point, the heart suddenly began to beat at about half its former rate. Ellis claims to have demonstrated that blood capillaries on the afferent (cardiac) side of the gills became gorged with blood, while on the efferent side, blood flow almost ceased. He concluded that the mucus prevented contraction of the gill filaments, this being necessary to maintain normal blood circulation. In the complete absence of histological data, this inference must be considered inconclusive.

The behaviour of the ten-spine stickleback (Pygosteus pungitius), when exposed to lethal concentrations of zinc

sulphate and other fish poisons, has been studied by Jones (1947b). The fish avoided concentrations of zinc down to 10 p.p.m., which is about thirty times the lethal threshold concentration. They did not avoid lower (but still lethal) concentrations. The fish avoided high concentrations of several other poisons, including copper sulphate, but they could not detect 32 p.p.m. copper and subsequently succumbed to it. In general, fish only avoided rapidly lethal concentrations of poison.

A recent study by Sprague (1964b) on the avoidance of zinc and copper salts by Atlantic salmon parr has produced strikingly different results from those of Jones. In the laboratory, parr avoided 9% of the lethal threshold concentration of zinc, 5% that of copper, and 2% that of an equitoxic solution of zinc and copper. In nature, adult salmon avoided 40% of the lethal threshold concentration of a zinc-copper mixture. Sprague has recently observed that young rainbow trout also avoid zinc and copper well below the lethal threshold concentration (Sprague, personal communication, 10 June 1964).

It is not clear whether these differing results by Jones and Sprague were caused by differences in experimental method, or whether they indicate great differences in behaviour between various test animals. If Sprague's results

prove to be of general application, they will be of great practical value, because they may be used to predict that the limiting level of pollution affecting a fishery will be the avoidance threshold concentration of a poison rather than the lethal threshold concentration.

Crandall and Goodnight (1962) have commented on physiological or behavioural changes in fish restricted to chronic concentrations of zinc. In 1.15 p.p.m. zinc, under conditions described in Section 2c (i), guppies were less active than the controls, ate less, swam abnormally and had difficulty in maintaining equilibrium. Similar observations were made on guppies held in lead nitrate and sodium pentachlorophenate solutions, except that these fish had normal appetites.

(iii) Accumulation of zinc

Radioactive zinc has been detected in marine fish following nuclear explosions in the Pacific area (e.g. Saiki, Okano, & Mori, 1955). Subsequently, the absorption of zinc by aquatic animals has been demonstrated by several workers. In all but one study, the zinc was labelled with zinc-65.

Saiki and Mori (1955) exposed clams (Meretrix meretrix) and carp (Cyprinus carpio) to low concentrations of zinc in sea water and fresh water respectively. The test animals were cultured in zinc solutions for periods up to 22 days.

Zinc was detected mainly in the gills, mantle and viscera of clams, but 40% of it was lost within 2 days after return to zinc-free sea water. Most of the zinc absorbed by carp was divided equally between gills and kidney. When zinc was injected into the muscle of carp, however, most was detected in the kidneys and little in the gills.

Cairns and Scheier (1957) exposed bluegills (Lepomis macrochirus) to water containing 1 p.p.m. zinc. After 1 hour's exposure, zinc was detected in mucus covering the surface of the fish. After 24 hours, most of the zinc (and the mucus) had been precipitated to the bottom of the container, but some of the zinc had been absorbed by the fish.

Lloyd (1960) reported that two rainbow trout killed by a 20 p.p.m. solution of zinc contained respectively 7.4 and 12 p.p.m. zinc, wet weight. The concentration of zinc measured in the whole fish was therefore only half the concentration of zinc in the water. The gills of these fish contained 63 and 60 p.p.m. zinc, wet weight - three times the concentration in solution. In another experiment, Lloyd determined by an unstated method that the concentration of zinc in the gills of two other trout, also killed by a 20 p.p.m. solution of zinc, was 1,405 and 1,265 p.p.m. respectively, dry weight. The method of drying was not reported.

Joyner (1961) studied in detail the uptake and retention of zinc by starved brown bullheads (Ictalurus nebulosus), during and after non-lethal treatments with zinc chloride. Groups of three fish, weighing 8 grams per group, were exposed for 96 hours to 2-litre samples of lake water (hardness 75 p.p.m. $CaCO_3$) containing initial concentrations of 0.25, 0.50, 1.0, 3.0, and 6.0 p.p.m. zinc. The fish:zinc ratios were within the range of 16,000:1 to 660:1. The actual concentrations of zinc in the solutions during exposure of the fish were not reported, but due to the high fish:zinc ratios they were probably much lower than the initial concentrations. (Compare with Cairns and Scheier's work.) The rate of uptake was first rapid and then slow in all treatments, but this may have been due to the diminishing concentrations in the solutions. The concentrations of zinc in the whole fish (wet weight) never exceeded the initial concentrations in the solutions in which the fish were immersed. Over half the total amount of zinc absorbed by the fish after 96 hours was detected in the gut and gills, with decreasing quantities in liver, kidney, skin, muscle, bone, and spleen. Bullheads exposed for 96 hours, to a solution that contained initially 6 p.p.m. zinc, lost 43% of their total accumulated zinc after 24 hours in zinc-free lake water. At the end of a further 6 days, only 11% more of the zinc had been lost.

Joyner and Eisler (1961) exposed salmon fingerlings (Oncorhynchus tshawytscha) to a concentration of 0.2 p.p.m. zinc in lake water for 24 hours. Nearly all the zinc was retained in the fish for 63 days. Its initial location was not determined, but after a week much of it was detected in the bones. The authors suggested that the bone surface acted as an ion-exchange bed that readily absorbed the zinc. Later, the overgrowth of normal bone tissue in the rapidly growing fish sealed the zinc in and isolated it.

Slater (1961) studied the accumulation of zinc by three species of trout; rainbow trout (Salmo gairdneri) cutthroat trout (S. clarki) and brook trout (Salvelinus fontinalis). Single, starved, juvenile fish were immersed for 48 hours in 200 ml. of lake water containing 0.37 p.p.m. zinc. The fish:zinc ratio apparently ranged from 8,400:1 to 42,000:1. Unlike the bullheads in Joyner's (1961) experiments, the trout accumulated zinc at approximately the same rate for 48 hours, in spite of the high - and necessarily increasing - ratio of fish to zinc in solution. Brown trout absorbed the most zinc and rainbow trout the least.

All investigations have shown that zinc entered fish exposed to zinc salts in solution. The concentrations of zinc which entered the whole fish, on a wet weight basis, were in every case lower than the concentration of zinc in the

surrounding medium (Joyner, 1961; Lloyd, 1960). Higher concentrations were localized in gill, kidney and gut. Labelling with zinc-65 has shown how much zinc entered a fish exposed to a solution of zinc, but it has not been used to assess the concurrent loss of zinc previously in the fish's body. The relative importance of exchange and accumulation of zinc in fish exposed to a solution of zinc has so far not been investigated.

Mount (1964) has demonstrated by polarography that five species of fish killed by rapidly lethal concentrations of zinc had a greater proportion of zinc in the gill than in the opercular bone, compared with control fish. The gill:bone ratios of zinc concentration, in fish exposed to non-lethal concentrations of zinc for up to 90 days, could not be distinguished from those of control fish, although the absolute values of zinc concentration were much greater. Mount has thus discovered a practical method for identifying acute zinc poisoning as a cause of fish mortality.

(iv) Entry of zinc

The possible routes by which zinc compounds may enter the bodies of aquatic animals are the gills, body surface, and alimentary canal. Jones (1939c) assessed their relative importance to freshwater fish in the following passage (p.432).

> It is generally believed that the integument of the teleost fish is completely impermeable to dissolved salts, but the fact that freshwater fish normally swallow very little water, though their urine is dilute and copious, seems to imply that they absorb quantities of water through the gills and the lining of the mouth cavity as suggested by Smith (1930), though whether salts enter the body in this way is uncertain.

In 1965, little more can be said than that. Joyner (1961) demonstrated that when the oesophagus of the brown bullhead was plugged with petroleum jelly, the fish absorbed as much zinc as untreated fish. In both groups, the gut wall contained a high concentration of zinc. Joyner believed that zinc entered the fish through both the gills and the skin. In particular, he suggested that zinc combined with mucus secreted by the skin, and was subsequently taken up by the fish from the mucus. Concerning the latter route, Cairns and Scheier (1957) detected zinc in the mucus secreted by bluegills that had been exposed to a solution of zinc ions. Most of the zinc - and mucus - was later precipitated on to the bottom of the test tank. Saiki and Mori (1955) found that carp immersed in a zinc solution contained a large amount of zinc in the gills, but carp injected intramuscularly with zinc contained very little zinc in the gills. Thus, there is

some direct evidence that zinc is not absorbed by fish through the gut, slight direct evidence that passage through the skin is unlikely, and meagre indirect evidence that infiltration through the gills may be substantial. Concerning invertebrates, the only comment has been by Anderson (1948), who suggested that the resistance of Daphnia magna to poisons decreases at ecdysis because the exoskeleton may then be more permeable.

(v) Hypotheses concerning toxic action

A number of hypotheses have been proposed to explain how zinc compounds kill aquatic animals, especially fish. These are as follows:

1. Coagulation of mucus on the gills of fish causes the breakdown of certain vital processes, particularly gas exchange, nitrogenous excretion, salt balance, and circulation of the blood.
2. Cytological damage to the gills of fish causes similar breakdowns.
3. Zinc coagulates protoplasm, following its absorption into the bodies of aquatic animals.
4. Long exposure of fish to low concentrations of zinc subjects them to stress, which induces adverse changes to essential organs resulting in death.

The opinions of the principal workers in the field of zinc toxicity, concerning the toxic action of zinc, will now be outlined.

Carpenter (1927, p. 390; 1930, p. 407) believed that lethal concentrations of zinc, lead, copper and cadmium salts kill fish by suffocation induced by mucus coagulated on the gills. Carpenter did not believe that heavy-metal ions actually penetrate the body.

The same view was shared by Jones (1938, p. 406; 1939c, p. 435; 1947a, p. 310) on the basis of his work on zinc, lead, and copper salts. He further suggested that the action of salts of the alkaline earths antagonizes the action of salts of the heavy metals by preventing coagulation of mucus. Suffocation occurs when coagulation proceeds faster than the secretion of fresh mucus. Concerning tadpoles and Polycelis nigra (a planarian), Jones suggested (1939a, p. 332; 1940a, p. 415) that heavy-metal salts penetrate the body and coagulate protoplasm either generally or selectively.

Ellis (1937, pp. 401-2) proposed all of the variations of Hypotheses 1 and 2 listed at the beginning of this section. He suggested that high concentrations of salts of the heavy metals, and also various other unspecified compounds, kill fish by anoxaemia, carbon-dioxide retention and circulatory collapse, following the clogging of the gills

with precipitated mucus and direct damage to the gill cells. He further suggested that more dilute solutions of heavy-metal salts might poison the gill cells, rendering them unable to excrete chloride ions and nitrogenous wastes.

Lloyd (1960, p. 91) concluded from his work that zinc sulphate acts specifically on the gills of fish, and does not act as an internal poison. Lloyd did not consider that mucus interfers seriously with gill function. In a later paper (Lloyd, 1962) he proposed the following sequence of events:

> When the rate at which (zinc) ions enter the gill epithelial cells is less than the rate at which they are removed into the blood stream, no build up of metal ions will occur in the epithelial cells and the fish will survive. If the rate at which (zinc) ions enter the gill epithelium is greater than the rate at which they are removed ...then a build-up will occur and the fish will die.

Lloyd had no specific suggestions concerning the immediate cause of death.

Crandall and Goodnight (1963, p. 71) suggested that the prolonged exposure of fish to low levels of zinc sulphate or other poisons subjects them to stress. This causes a hormonal imbalance which induces a variety of pathological changes. In addition, normal growth and maturation are inhibited, possibly by inadequate food intake but more probably by poor food utilization. A combination of numerous adverse changes

results in general enfeeblement and ultimately in death.

Clearly, several of the opinions just outlined are contradictory. The four hypotheses proposed at the beginning of the present section will now be evaluated in the light of our present knowledge. Finally, some generalizations will be made about the toxic action of zinc.

Concerning Hypothesis 1, information about the coagulation of mucus on fish gills by acutely toxic concentrations of zinc salts is conflicting (Section 2c (i)). Carpenter (1927) and Jones (1938) both observed that zinc coagulates mucus on the gills and Lloyd (1960) that it does not. Only Lloyd supported his observation by histological data. Moreover, no-one has effectively demonstrated that coagulated mucus, from whatever cause, interferes in any way with any vital function of fish, although Ellis' interesting observation about heart beat has been noted (Section 2c (ii)).

Concerning Hypothesis 2, only Lloyd (1960) has demonstrated by histology that cellular damage to the gills occurs when fish are exposed to a zinc salt, although three other workers (Section 2c (i)) have shown that a variety of other compounds have the same effect on fish. No-one has shown that gill damage causes death through breakdown of any vital gill function, although it is usual and reasonable to presume

that it does. It would be interesting to know which essential function breaks down first when either the gills become damaged, or covered with coagulated mucus, or when both these changes occur together. Presumably, if the gill epithelium were sloughed off without the gills becoming clogged, gas exchange might even be facilitated, because the red blood cells would then be separated from the oxygen in solution only by the capillary walls. In this case, death might result primarily from an upset of salt balance. This idea could be readily tested by comparing the survival of poisoned fish in tap water and a suitable, isotonic, salt solution.

Evidence supporting the third hypothesis is quickly accounted for: there isn't any! A succession of workers have shown that zinc is absorbed by fish and clams, both from lethal and non-lethal concentrations (Section 2c (iii)). However, none of them have observed any coagulated protoplasm in any aquatic animal.

The fourth hypothesis is supported by the evidence of numerous cellular changes described by Crandall and Goodnight (Section 2c (i)). These changes are similar to those occurring in fish and higher vertebrates subjected to various kinds of stress (e.g. Rasquin & Rosenbloom, 1954; Selye, 1950). The sequence of events occurring in vertebrates exposed to stress has been summarized by Deevey (1959). The

histological changes noted by Crandall and Goodnight correspond well to Deevey's description of the later stages of the stress syndrome. Unfortunately, Crandall and Goodnight did not extend their study to examine the interrenal tissue (which is homologous to the adrenal cortex of mammals), and the other endocrine glands. It is changes in these tissues which are believed to trigger off the widespread modifications of the type noted by Crandall and Goodnight.

Summarizing this section so far, the support for the above four hypotheses is as follows. The 'coagulated - mucus' hypothesis is possible but unproven in those situations where mucus is precipitated. The 'gill - damage' hypothesis is probable but unproven in acutely toxic concentrations of zinc. The 'coagulated - protoplasm' hypothesis is untested. The 'stress' hypothesis is probable and partly proven where fish are exposed to chronically toxic concentrations of zinc.

From the little that is known about the toxic action of zinc compounds, three generalizations concerning fish appear fairly safe. The first is that toxic action varies with concentration. At acutely toxic concentrations, zinc appears to act primarily on the gills of fish, although it should be remembered that histological changes have not been looked for elsewhere. At chronically toxic concentrations, zinc causes

no damage to the gills, but widespread changes occur in many other organs.

The second generalization is that toxic action of zinc varies with life history. Fish embryos cannot die from gill damage until they have developed gills. Reliable information about fish eggs and newly hatched fish is however entirely lacking.

The third generalization is that the toxic action of zinc is non-specific. Gill damage in adult fish is caused by a selection of unrelated compounds. Stress-induced changes are apparently caused by a variety of adverse situations, most of them non-toxic. On the other hand, zinc poisoning can be positively diagnosed by examining the gill:bone ratio of zinc concentration in dead fish.

## 3. RESISTANCE OF THE ZEBRAFISH TO ZINC SULPHATE

### a) Materials and methods

#### (i) Choice of test animal

I chose the zebrafish (*Brachydanio rerio*), a member of the Family Cyprinidae, as a convenient test organism because it is small, easy to rear and breed in small tanks at constant temperature (Legault, 1958; Sterba, 1962), has a life cycle of only a few months, and lays non-adhesive eggs. A single pair of adults will frequently produce 600 eggs at a single laying, and development until hatching has been described (Hisaoka & Battle, 1958). Eggs hatch during the third day at 25°C. The hatched fish will survive without feeding until the thirteenth day and become sexually mature at about 100 days.

#### (ii) Rearing conditions

All test fish were largely reared, and all experiments were carried out in a mixture of Canberra tap water and distilled water, at a total hardness of $10 \pm 2$ p.p.m. as $CaCO_3$, a temperature of $25 \pm 2$°C., pH between 6.8 and 7.2, and

dissolved-oxygen concentration at least 6 p.p.m. The total hardness was due mainly to calcium and magnesium ions in approximately equal proportions. The results of some chemical analyses of Canberra tap water, during the period of this study, are presented in Table 4. An acceptable level of dissolved oxygen was maintained without aeration, except where otherwise noted.

All fish more than 13 days old were maintained at the prescribed temperature, total hardness, pH and dissolved-oxygen concentration for at least 10 days before use in toxicity bioassays. Prior to this acclimatization period, the hardness of the water was not strictly controlled, but never exceeded 30 p.p.m. $CaCO_3$. Other conditions were as described. Younger fish were maintained under the prescribed conditions from time of laying. Fish more than 13 days old were fed daily on a variety of foods which included the following ingredients:- living and frozen daphnids and cyclops, dried liver and shrimp, and 'Fiesta' tropical-fish food and goldfish food. They were starved for 24 hours prior to use in bioassays. Test fish less than 13 days old were not fed at all. If they were intended for bioassays after the thirteenth day, they were fed on 'Liquifry' liquid foods and on microscopic green algae. No fish more than 13 days old died during the acclimatization period. The mortality of 13-day-old fish was

Table 4. Some analyses of five samples of Canberra tap water, collected between June 1963 and February 1964.

| Factor | Mean value | Range |
|---|---|---|
| Total solids (p.p.m.) | 38 | 35-43 |
| Total alkalinity (as p.p.m. $CaCO_3$) | 15 | 13-18 |
| Total hardness (as p.p.m. $CaCO_3$) | 13 | 11-15 |
| calcium (as p.p.m. $CaCO_3$) | 7 | 6-9 |
| magnesium by subtraction (as p.p.m. $CaCO_3$) | 6 | 5.4-9 |
| Total iron (p.p.m. Fe) | 0.4 | 0.2-0.7 |
| Copper (p.p.m. Cu) | nil | - |
| Chemical oxygen demand (as p.p.m. O) | 1.4 | 0.9-2.7 |
| Chloride (p.p.m. Cl) | 2.1 | 0.8-3.5 |
| Sulphate (p.p.m. $SO_4$) | nil | - |
| Free chlorine (p.p.m. $Cl_2$) | 0.1 | nil-0.2 |
| Turbidity (Jackson - candle units) | 3 | 1.5-4 |
| pH | 7.0 | 6.9-7.1 |

about 20%, many fish dying probably from starvation. The mortality of younger fish was in every case less than 10%.

As far as possible, all the test animals in a single series of experiments were randomly drawn from a population of fish which were the progeny of a single spawning of a pair of zebrafish. A different pair of parents was used for each series. All the six parents were drawn from a single stock of zebrafish that had been pond-reared in Hong Kong by Union Aquariums, Ltd., of Kowloon. All toxicity bioassays with adults were conducted with pond-reared fish that were drawn from the same stock as the six parents and subsequently acclimatized in tanks in the laboratory.

(iii) Exposure to zinc

In three preliminary experiments, six concentrations of zinc were tested:- 1.3, 2.5, 5, 10,20 and 40 p.p.m. as Zn. Because zebrafish of all ages survived a considerable time in the two lowest concentrations, the main study was restricted to the four highest concentrations, in order that the test fish would die before their age could change significantly. The amounts of zinc in solution never differed from the intended values by more than 10% in the four highest concentrations, nor by more than 25% in the two lowest concentrations. Zinc was absent from the tap water.

All bioassays were carried out using approximately five test animals in each test container. Fish up to 13 days old were exposed to 60 ml. of solution in small culture dishes, 40-day-old fish to 1 litre of solution in large culture dishes, and 100-day-old fish to 6 litres of solution in plastic buckets. The mean wet weights of individual 1-day-old eggs, 6-day-old hatched fish, 40-day-old fish and 100-day adult fish were 1.1, 0.3, 26 and 320 mg., respectively. If the test fish survived 3 days, 90% of the test solution was replaced on the third day, and thereafter weekly. Forty and 100-day-old fish were fed once daily during bioassays, starting on the second day.

The wet weights of samples of five test animals, per unit of weight of zinc sulphate (as Zn) in the test solutions, are shown in Table 5. The highest fish:zinc ratio (53:1) occurred when adult fish were exposed to 5 p.p.m. zinc. The greatest drop in concentration of zinc in a test solution during a bioassay also occurred, as expected, when adult fish were exposed to 5 p.p.m. zinc. The loss in zinc from the test solution was 0.2 p.p.m., or 4% of the total. Thus, at the fish:zinc ratios used, the effect of the test animals in lowering the toxicity of zinc sulphate is considered to have been negligible.

Table 5. Wet weight of samples of five test animals per unit weight of zinc sulphate (as Zn) in test solutions.

| Age of fish | Concentration of zinc sulphate in solutions (p.p.m. Zn) | | | |
|---|---|---|---|---|
| | 40 | 20 | 10 | 5 |
| 0-3 days | 2.3 | 4.5 | 9.0 | 18 |
| 4-13 days | 1.0 | 2.0 | 3.9 | 7.7 |
| 40 ± 5 days | 3.3 | 6.6 | 13 | 26 |
| approx. 100 days | 6.6 | 13 | 26 | 53 |

In each of the three preliminary experiments, two batches of five test fish were exposed to each of the six test concentrations of zinc. Ten control fish were maintained in tap water. The ages of the animals in the three experiments were 0.1, 6.1 and 40 ± 5 days, respectively. Bioassays were terminated after 7 days.

In any one experiment in the main study, two batches of approximately five fish were exposed to each of the four highest test concentrations, and again ten control fish were maintained in tap water. Therefore about fifty fish were used in each experiment. Bioassays were continued if necessary for at least 21 days. This basic procedure was followed with zebrafish of eleven different ages:- unhatched embryos aged 0.1, 1.1, 2.1 and 3.1 days; hatched fish aged 4.1, 6.1, 8.1, 10.1, 13.1 and 40 ± 5 days; and sexually mature adults aged approximately 100 days. This series of experiments was carried out three times, so that finally about thirty fish of each age group were exposed to each of the four treatments. The main study therefore consisted of thirty-three experiments in which approximately 1,650 fish were used.

(iv) Analytical methods

The bioassay solutions were analysed before the test animals were added, after most of them had died, and whenever

test solutions were changed. Total hardness was measured by titration with 0.005 M ethylenediaminetetraacetic acid or EDTA (American Public Health Association, 1960). Zinc in solution was usually determined by back-titration of the resulting solution with a 0.005 M calcium solution, after the addition of potassium cyanide (Schwarzenbach, 1957). For both tap water and the two most dilute test solutions, zinc concentrations were determined polarographically by the method of standard addition, using a Cambridge Universal Polarograph. The accuracy of some of the titrimetric analyses of zinc were also confirmed by the same polarographic method. Dissolved-oxygen levels were determined by the Alsterberg modification of the Winkler method (APHA, 1960), and pH by using a standard instrument.

(v) Estimation of survival time

The end point of each toxicity bioassay using hatched fish was the cessation of fin and gill movements, and a lack of response by the animal when touched. Unhatched embryos were examined microscopically to determine immobilization. Embryos more than 24 hours old were considered to be immobilized when tail movements and heart beat ceased. With embryos less than 24 hours old, arrest of development was taken as the end point. Although these various criteria may not be

exactly comparable, in practice it was found that when embryos and hatched fish showed any of the end points described above, the tissues turned opaque within an hour. Decay was retarded by the zinc in solution, but was rapid upon transfer of the dead animals to tap water.

The survival times of individual test animals were determined by inspection after suitable periods of exposure, which increased more or less logarithmically. Intervals between inspections were sufficiently short to permit the calculation of survival times with an error of not more than 10%. For example, if a fish were alive after 9 hours' exposure and immobilized after 11 hours, its survival time would be estimated to be $10 \pm 1$ hours - an error of 10%. This standard of accuracy could not be maintained during the first night of a bioassay. With the few fish that died during this period, the error was not more than 20%. Mortality of the control fish never exceeded 10% in any experiment. None of the control fish aged more than 13 days died during a toxicity bioassay.

The median survival time of test animals of the same age and series, and exposed to the same treatment, was usually estimated by probit analysis (Bliss, 1937). A typical result is shown in Fig. 43. The median survival time of all thirty animals of the same age, that were exposed to the same

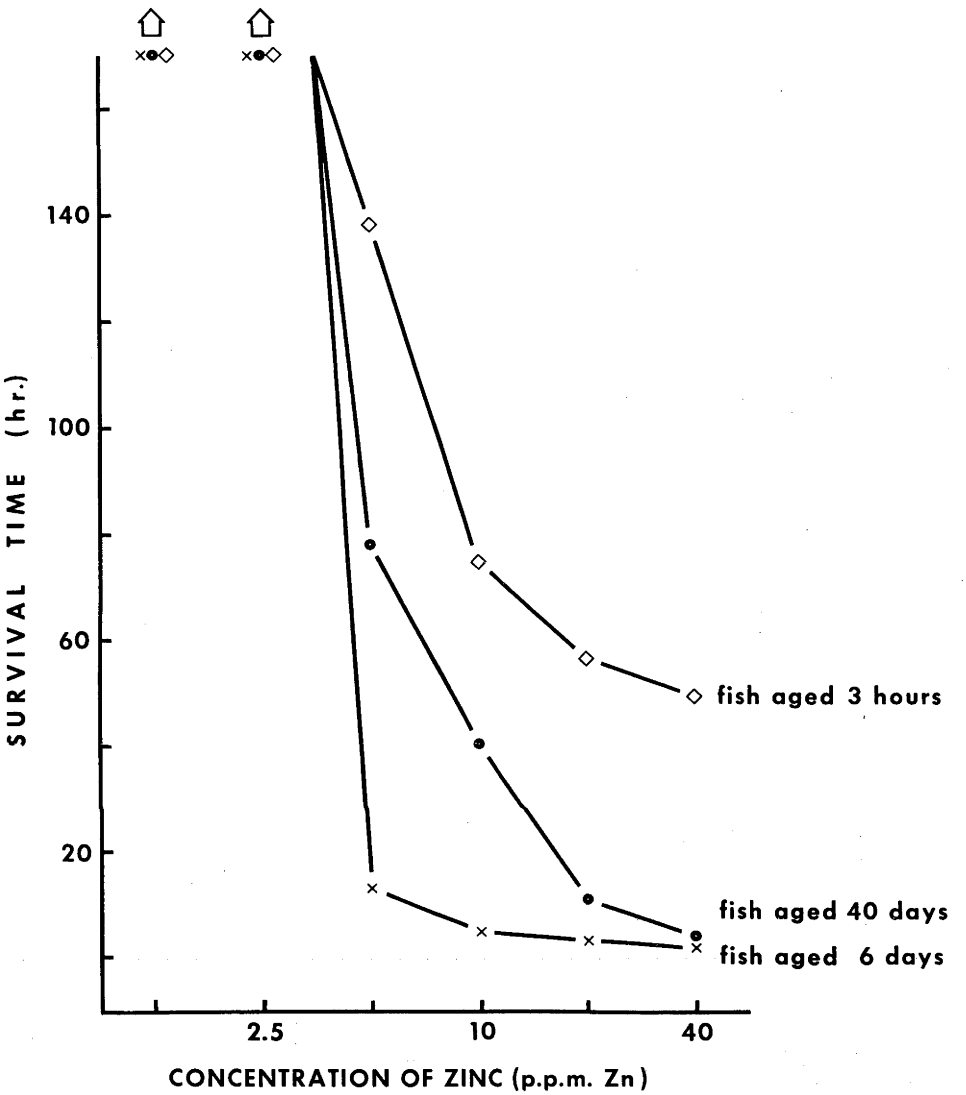
treatment, was then estimated by taking the arithmetic mean of the median survival times for the three series. The median survival time of adult fish that were exposed to 5 or 10 p.p.m. zinc, and of 40-day-old fish exposed to 5 p.p.m. zinc could not be estimated because cumulative mortality was less than 50%. This difficulty is illustrated in Figs. 44 and 45, and discussed in Section 7c (ii).

b) Results

The results of the three preliminary experiments are presented in Fig. 4. In the four highest concentrations of zinc, 3-hour-old embryos proved to be the most resistant age group, as is shown by their surviving the longest time in any given concentration. Six-day-old fish survived the shortest time. The lethal threshold concentrations in all three experiments, that is to say the highest concentrations which just failed to kill under prolonged (theoretically infinite) exposure, appeared to lie between 2.5 and 5 p.p.m. zinc. Thus the threshold concentrations of zinc were approximately the same for all three age groups, in spite of great variations in resistance at higher concentrations.

As these estimates of threshold concentration were based on an exposure time of only 7 days, they are probably higher than the true values for the species. This was in fact found

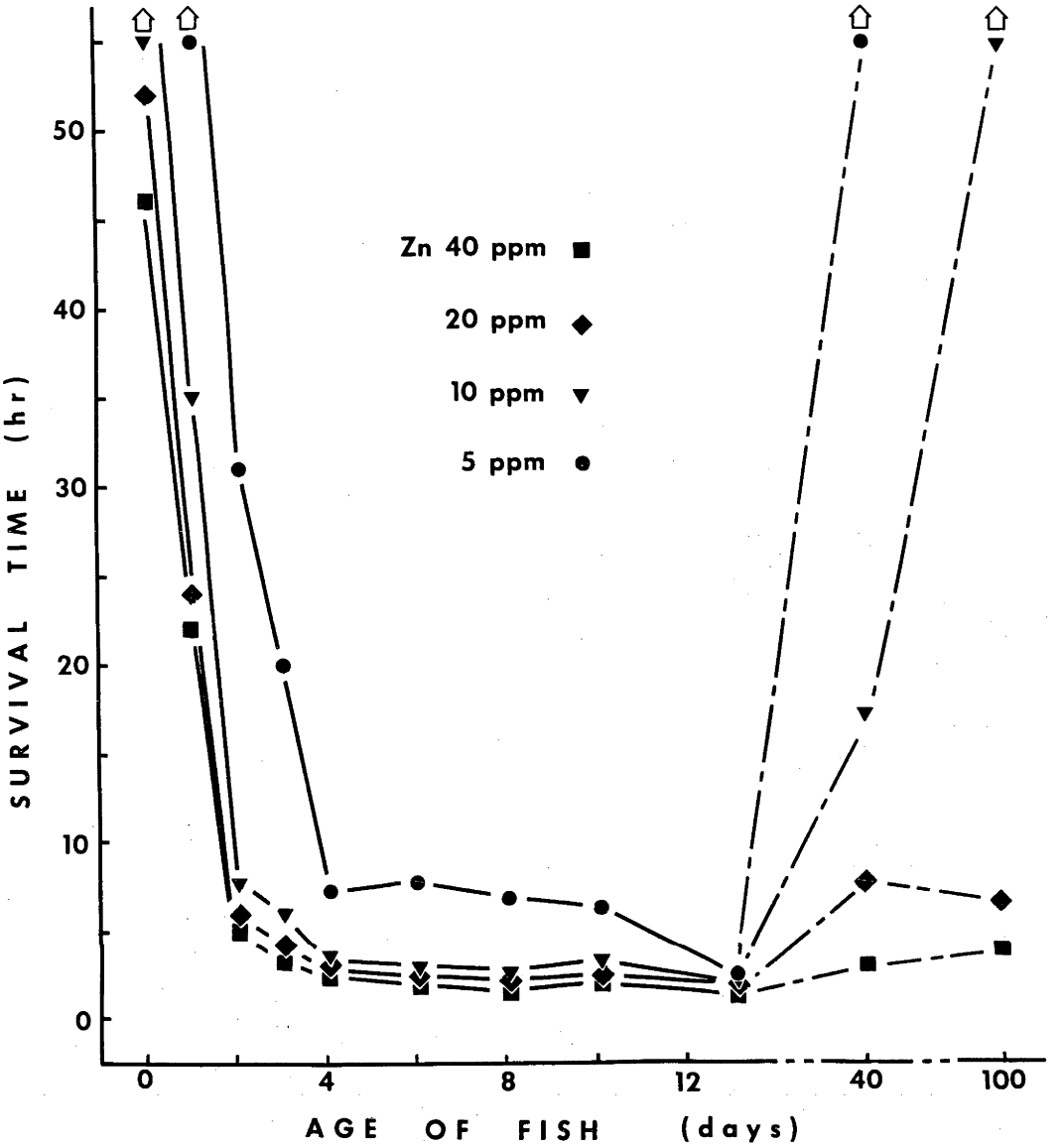
Fig. 4. Survival of zebrafish of three different ages, when exposed to six concentrations of zinc sulphate in Canberra tap water (total hardness 10 p.p.m. $CaCO_3$), at 25°C., pH of 7.0, and an oxygen concentration of more than 6 p.p.m. Each point represents the median value for ten fish.


SURVIVAL TIME (hr.)
140
100
60
20
2.5
10
40
CONCENTRATION OF ZINC (p.p.m. Zn)
fish aged 3 hours
fish aged 40 days
fish aged 6 days

to be the case with 40-day-old fish. In a histological study, which will be described in Section 7, samples of about a dozen 40-day-old fish were exposed to 6-litre quantities of water, containing 2.5, 1.3 or 0 p.p.m. zinc, respectively. Bioassay conditions were otherwise similar to those described here. None of the fish in the tap water died of natural causes. Median survival times were 15 days in 2.5 p.p.m. and 39 days in 1.3 p.p.m. zinc. The lower concentration is believed to be slightly above the true threshold value because only two out of eleven test fish survived for 116 days in 1.3 p.p.m. zinc, and they were very stunted (Table 15). Prolonged exposures of eggs and newly hatched fish to low concentrations of zinc were not attempted. It will be shown later in this section that the threshold concentration for adult fish appeared to be about 10 p.p.m. zinc, suggesting that the threshold increases with age.

The results of the thirty-three experiments of the main study are presented in Fig. 5, each point representing the median survival time of approximately thirty animals. The four curves demonstrate clearly that newly laid eggs were highly resistant to zinc poisoning. Resistance decreased steadily with age until the fourth day, that is until just after hatching. From the fourth to the tenth day, the resistance of hatched fish was uniformly low, and slight

Fig. 5. Survival of zebrafish of eleven different ages, when exposed to four concentrations of zinc sulphate in Canberra tap water (total hardness 10 p.p.m. $CaCO_3$), at 25°C., pH of 7.0, and an oxygen concentration of more than 6 p.p.m. Each point represents the median value for approximately thirty fish.


SURVIVAL TIME (hr)
50
40
30
20
10
0
Zn 40 ppm
20 ppm
10 ppm
5 ppm
0
4
8
12
40
100
AGE OF FISH (days)

fluctuations in the curves were probably due to sampling errors. Resistance was lowest at the thirteenth day, soon after which unfed control fish died, probably from starvation. At the four concentrations of zinc tested, newly hatched fish aged from 4 to 13 days formed by far the most sensitive stage in the life history. At concentrations of 20 and 40 p.p.m. zinc, the resistance of 40 and 100-day-old fish was slightly greater than that of 4 to 13-day-old fish, but considerably lower than that of unhatched embryos. At concentrations of 5 and 10 p.p.m. zinc, 40-day-old fish were more resistant than newly hatched fish, many individuals surviving for several days. At the same concentrations, 100-day-old fish were highly resistant, 96% of twenty-five fish surviving 5 p.p.m. for 21 days and 55% of twenty-nine more fish surviving 10 p.p.m. for the same period. After 21 days, all the survivors appeared to be in good condition, eating well and swimming normally. The survivors of the earliest experiment with adult fish were still vigorous after 84 days in the test solutions, and some of them spawned following their return to tap water (Section 7c (i)). Deaths after this time were attributed to old age.

It is apparent from data discussed in the previous paragraph that the resistance of zebrafish to zinc poisoning varied widely with life history, newly laid eggs being most

resistant and newly hatched fish being highly susceptible. This interesting result could not be predicted from the literature (Section 2b (viii)) because information on changes in resistance with life history were almost entirely lacking. Jones (1938) observed no difference in resistance to zinc poisoning between juvenile and adult sticklebacks. Anderson (1948) attributed variations in resistance of daphnids to changes in permeability associated with ecdysis, an event not parallelled in fish. It is however significant that Mathews (1904) found the eggs of Fundulus (a marine teleost) to be highly resistant to zinc poisoning.

c) Hypotheses concerning resistance

It has been established in the previous section that the resistance of zebrafish to zinc poisoning varies with age. In the remainder of this thesis, an attempt will be made to determine why this should be. Four hypotheses will be considered in turn, each of which attempts to correlate resistance with some other readily observable factor, either throughout life history or at a particular phase of it.

Hypothesis 1 is that the high resistance of unhatched zebrafish embryos to zinc sulphate is owing to protection afforded by the chorion.

It has been observed that resistance decreases steadily with age from the time the egg is first laid until time of hatching, and then remains substantially the same for several days. An obvious interpretation of these data could be that the resistance of the unhatched embryo is high because the membrane surrounding the embryo - the vitelline membrane or chorion - protects the embryo, either by excluding the zinc or in some other way. As permeability increases with age, resistance declines. The hypothesis is supported by the observation that newly laid eggs possess a flexible chorion which is only pierced with difficulty, whereas the chorion of 2-day-old eggs is brittle and can be readily removed with a sharp needle and forceps. Most embryos hatch on the third day at 25°C.

A review by Smith (1957) of the literature on teleost eggs indicates that most of those studied are believed to possess a chorion which is largely impermeable to salts. This evidence suggests that the chorion may protect the embryo from a solution of zinc sulphate by keeping out the zinc.

The first hypothesis is tested in Section 4, by comparing the survival of embryos having the chorion undamaged with others in which the membrane is either ruptured or removed.

Hypothesis 2 concerns the relationship between resistance and rate of metabolism. The rate of oxygen uptake of fish has been generally taken to be a reasonable measure of their metabolic rate (Fry, 1957, p. 23), and the same assumption will be made here. It is known that the rate of oxygen uptake increases rapidly during early embryological development (e.g. Trifonova, 1937), while the resistance of the zebrafish at this stage has been shown to decline with age. Further, there have been three studies of the resistance of fish to poisons, in which oxygen uptake has also been measured. Resistance of several species of fish to various organic poisons has been shown to increase, and oxygen uptake to decrease, during acclimatization to new conditions (Sumner & Wells, 1935; Weiss & Botts, 1957). Herbert and Shurben (1963) observed that actively swimming trout survived a shorter period than resting trout in toxic solutions of zinc sulphate and ammonium chloride. The decrease in resistance with activity was found to be related to increase in oxygen uptake.

The second hypothesis is therefore proposed that the resistance of the zebrafish to zinc sulphate is inversely proportional to the rate of oxygen uptake of the fish. The hypothesis will be considered in Section 5.

Hypothesis 3 deals with zinc uptake. It is known that zinc is taken up by fish from the surrounding water (e.g. Joyner, 1961; Slater, 1961). A high concentration of zinc has been detected in the bodies of trout killed by zinc poisoning (Lloyd, 1960). It seems reasonable to suppose that the rate of uptake of zinc is related to general metabolism and thus to rate of oxygen uptake. The higher the rate of zinc uptake, the more rapid would be the toxic action of the zinc, the shorter would be the survival time of the fish, and the lower their resistance.

The third hypothesis is therefore proposed that the resistance of zebrafish to zinc sulphate is inversely proportional to the rate of whole-body zinc uptake. The hypothesis will be considered in Section 6.

Hypothesis 4 relates resistance with mode of toxic action. Histological examination of the gills of fish, which had been killed rapidly by a variety of poisons including zinc sulphate, revealed severe cytological damage (e.g. Lloyd, 1960; Christie & Battle, 1963). Gill damage has been generally accepted by these and other authors to be the specific cause of death. Previous studies have all been limited to adult and juvenile fish; but it is obvious that unhatched embryos could not suffer gill damage before they had developed gills. It may therefore be inferred that the toxic action of

rapidly lethal concentrations of zinc must vary with life history. The question is further complicated by the observation that chronically toxic concentrations of zinc sulphate and other poisons caused cytological changes in many organs but not in gills (Crandall & Goodnight, 1963). Clearly, toxic action must also vary with concentration.

The fourth and last hypothesis to be considered in this thesis is that variations in the resistance of zebrafish to zinc sulphate are associated with differences in the toxic action of zinc. Data concerning toxic action will be presented in Section 7.

## 4. EFFECT OF THE CHORION ON THE RESISTANCE OF UNHATCHED EMBRYOS

### a) Materials and methods

Zebrafish embryos of common parentage were bred and reared in Canberra tap water (total hardness 10 p.p.m. $CaCO_3$), at a temperature of 25°C., pH between 6.8 and 7.2, and dissolved-oxygen concentration at least 6 p.p.m., under conditions described in Section 3a. Mortality during rearing was less than 10%. Rates of development were similar to those described by Hisaoka and Battle (1958).

Thirty hours after laying, soon after the heart began to beat, the vitelline membrane or chorion was removed from fifty eggs, using fine scissors and forceps. The naked embryos were maintained in tap water for an additional 2 hours, to detect deaths through mechanical damage, or from other causes. None in fact occurred. Two 31-hour-old embryos are illustrated; one with its chorion removed (Fig. 6) the other with its chorion intact (Fig. 7).

Forty-two hours after laying, samples of ten naked embryos were transferred to each of four test solutions of zinc sulphate in tap water, containing 2.5, 5, 10 and 20

Fig. 6. 31-hour-old zebrafish embryo with chorion removed. (x40)

Fig. 7. 31-hour-old zebrafish embryo with chorion entire. (x40)

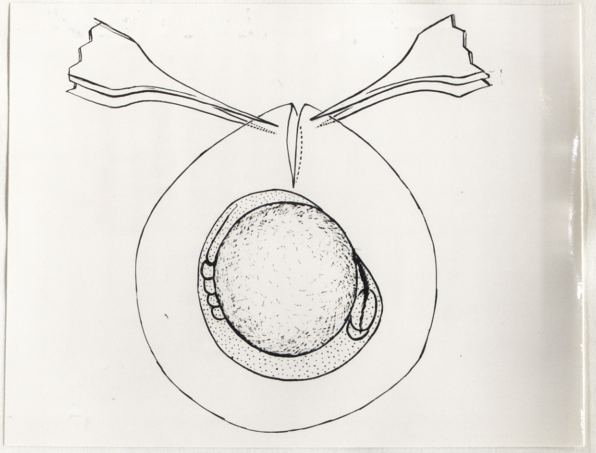
p.p.m. zinc respectively. Ten control embryos were maintained in tap water. Concurrently, samples of ten embryos with entire chorions (hereafter referred to as 'entire embryos') were transferred to solutions containing the same four concentrations of zinc and again ten control animals were set aside. Bioassay procedures were similar to those described in Section 3a. Individual times to immobilization were recorded with an error of less than 20%. None of the twenty control animals died.

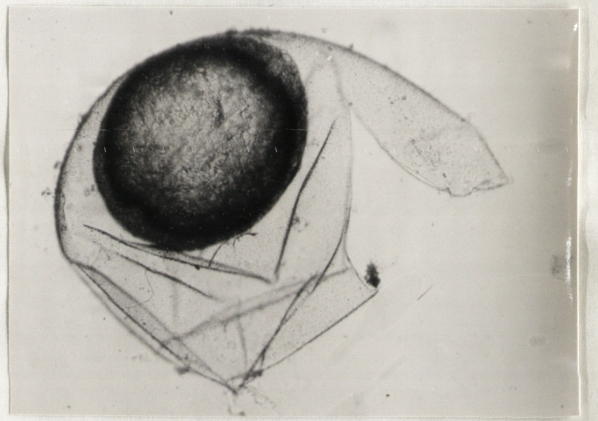
In a second experiment, a further batch of embryos were similarly bred and reared. Mortality was again less than 10%. Ten hours after laying, shortly after the closure of the blastopore, an unsuccessful attempt was made to remove the chorion using a variety of dissecting instruments. Owing to the toughness of the membrane, the embryos were damaged every time. Failing the complete removal of the membrane, it was ruptured in the following manner. A minute fold of membrane was grasped simultaneously with two fine forceps, and torn by pulling the forceps apart (Fig. 8). Care was taken to exert no pressure on the embryo. With practice, a tear could be made in the membrane, equivalent to approximately 10% of its total surface area. Forty-six eggs were successfully treated in this way between 10 and 13 hours after laying. They will be referred to later as

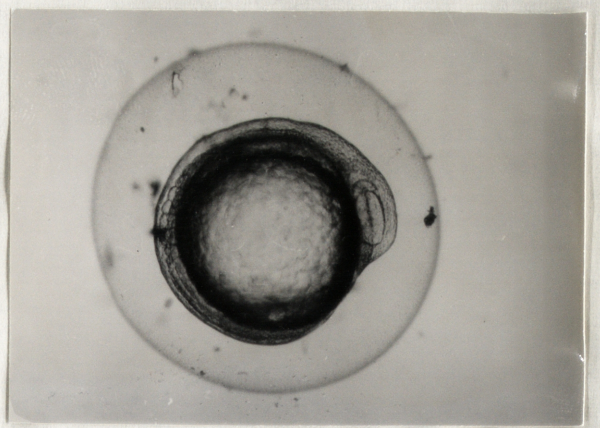
Fig. 8. Drawing of 13-hour-old entire embryo, showing method of rupturing the chorion. (x40)

Fig. 9. 14-hour embryo with chorion ruptured. (x40)

Fig. 10. 14-hour embryo with chorion entire. (x40)







'ruptured embryos'. Fourteen-hour-old ruptured and entire embryos are illustrated in Figs. 9 and 10, respectively. The forty-six ruptured embryos, plus forty-five more entire embryos, were maintained in tap water for several hours. None died.

Fifteen hours after laying, following development of the optic vesicle, twenty ruptured embryos and twenty-three entire embryos were transferred to a solution of zinc sulphate in tap water, containing 20 p.p.m. zinc. The rest of the embryos were retained in tap water. One of the twenty-six ruptured control embryos died. Twenty-five hours after laying, by which time twenty somites had developed, sixteen more ruptured embryos and twelve more entire embryos were transferred to the 20 p.p.m. zinc solution. Ten control animals of each group were maintained in tap water. Bioassay procedures were identical to those in the previous experiment. Individual survival times were noted as before, with an error of less than 20%. None of the twenty control animals died.

In a third experiment, yet another batch of embryos were bred and reared as before, with mortality again less than 10%. About 10 hours after laying, the chorions of seventy-seven embryos were ruptured, and the embryos held in tap water until 15 hours after laying. None died. Approximately 100 entire embryos from the same batch were also

maintained in tap water. None of them died. Fifteen and 25 hours after laying, samples of thirty ruptured embryos and thirty entire embryos were transferred to a 20 p.p.m. zinc solution in tap water. The individual survival times of all 120 embryos were noted, with an error of less than 10% in each case. Bioassay conditions were the same as before. None of the control fish died.

In all three experiments, the median survival times of samples of embryos exposed to similar conditions were estimated by probit analysis (Bliss, 1937). Median survival times of entire embryos and ruptured (or naked) embryos were then compared, all data from each experiment being considered concurrently. The statistical significance of differences between related medians was tested by the analysis of variance.

b) Results

The median survival times of samples of 42-hour-old embryos in four concentrations of zinc (Experiment 1) are shown in Fig. 11. The median survival times of samples of 15 and 25-hour-old embryos in 20 p.p.m. zinc (Experiments 2 and 3) are recorded in Table 6 and Fig. 12, respectively. (For purposes of comparison, the survival data of 42-hour embryos in 20 p.p.m. zinc have been repeated in Fig. 12 from Fig. 11.)

Fig. 11. Survival of entire and naked 42-hour-old zebrafish embryos, when exposed to four concentrations of zinc sulphate in Canberra tap water (total hardness 10 p.p.m. $CaCO_3$), at 25°C., pH of 7, and an oxygen concentration of more than 6 p.p.m. Each point represents the median value for ten embryos.

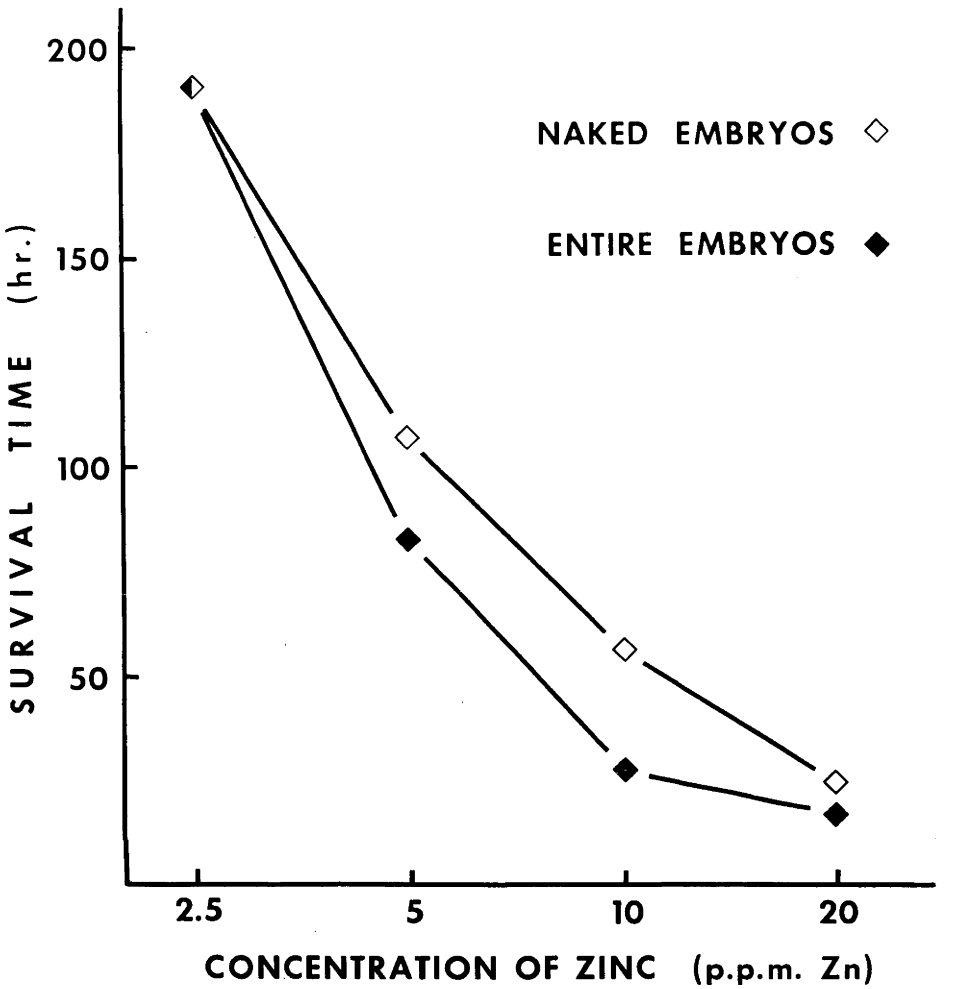

200
150
100
50
SURVIVAL TIME (hr.)
NAKED EMBRYOS
ENTIRE EMBRYOS
2.5
5
10
20
CONCENTRATION OF ZINC (p.p.m. Zn)

Fig. 12. Survival of entire zebrafish embryos, and naked (or ruptured) embryos, of three different ages, when exposed to zinc sulphate (20 p.p.m. Zn) in Canberra tap water (total hardness 10 p.p.m. $CaCO_3$), at 25°C., pH of 7, and an oxygen concentration of more than 6 p.p.m. For 15 and 25-hour-old embryos, each point represents the median value for thirty embryos. For 42-hour-old embryos, each point represents the median value for ten embryos. Data for 42-hour embryos from Fig. 11.

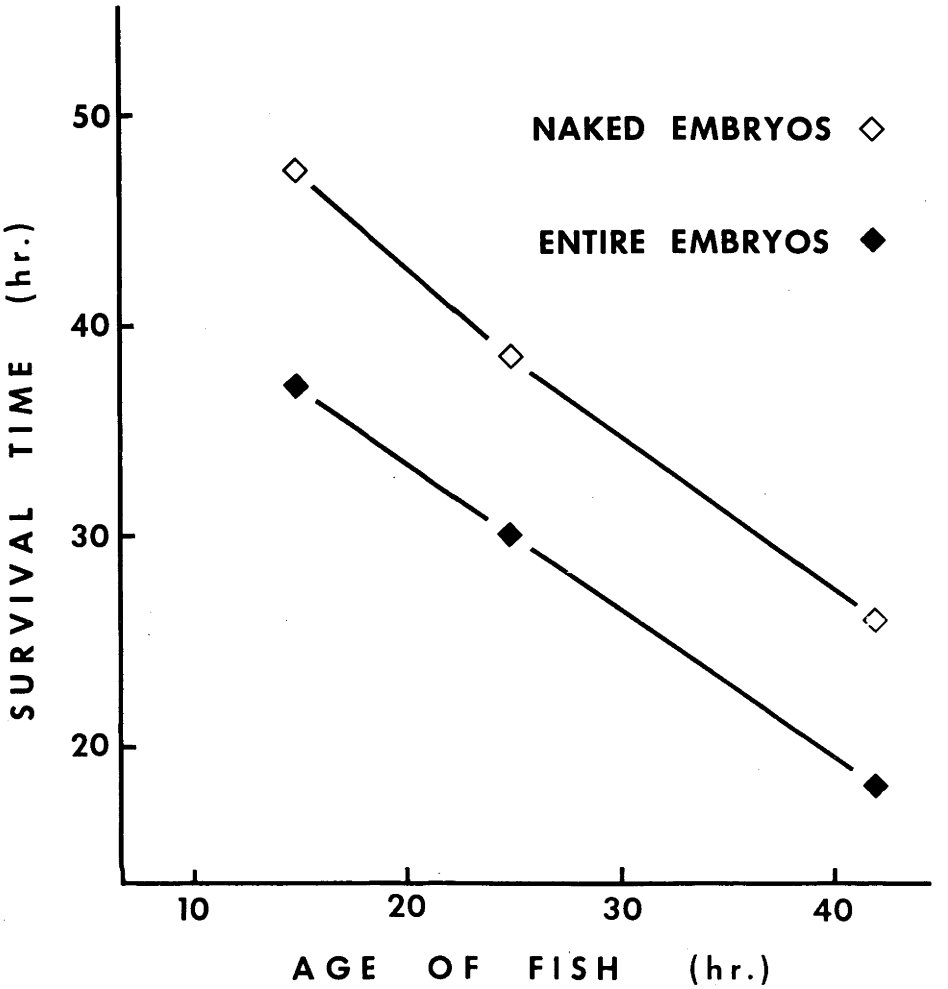

NAKED EMBRYOS
ENTIRE EMBRYOS
SURVIVAL TIME (hr.)
50
40
30
20
10
20
30
40
AGE OF FISH (hr.)

Table 6. Survival of ruptured and entire zebrafish embryos, of two different ages, when exposed to zinc sulphate (20 p.p.m. Zn) in Canberra tap water (total hardness 10 p.p.m. $CaCO_3$), at 25°C., pH of 7, and an oxygen concentration of more than 6 p.p.m. Sample sizes in parentheses.

| | Age of embryos at start | |
|---|---|---|
| | 15 hours | 25 hours |
| ruptured embryos | 50 hr. (20) | 34 hr. (16) |
| entire embryos | 47 hr. (23) | 31 hr. (12) |

The analysis of variance of data in Experiment 3 is listed in Table 7.

c) Discussion

(i) Consideration of Hypothesis 1

The prime purpose of this section is to consider Hypothesis 1 of Section 3c, which is that the high resistance of unhatched zebrafish embryos to zinc sulphate is owing to protection afforded by the chorion.

In Experiment 1, naked 42-hour-old embryos survived as long as entire embryos in 2.5 p.p.m. zinc (Fig. 11). This was the expected result because survival times extended far beyond hatching, after which event all embryos would naturally be naked. However, in all higher concentrations of zinc, naked embryos actually appeared to survive longer than those with the chorion entire! In Experiment 2, ruptured 15 and 25-hour-old embryos appeared to survive longer than entire embryos (Table 6).

These data demonstrate convincingly that the chorion did not protect the zebrafish embryo from the toxic effects of the zinc: the high resistance of the embryo to zinc poisoning was not owing to protection by this membrane. The first hypothesis must therefore be rejected. However, the study of

Table 7. Analysis of variance of data in Experiment 3.

| Source of variance | Degrees of freedom | Sums of squares | Mean squares |
|---|---|---|---|
| embryos* | 1 | 4,795 | 4,795 |
| groups+ | 1 | 1,235 | 1,235 |
| embryos x groups | 1 | 457 | 457 |
| replicate error | 116 | 27,877 | 240 |
| total | 119 | 34,364 | - |

* Embryos, ruptured or entire

\+ Age groups, 15 or 25 hours

For embryos: $F = 20.0$, $df = 1, 116$, $P < .001$

For groups: $F = 5.15$, $df = 1, 116$, $P = .025$

the chorion was continued to investigate the interesting possibility that its removal actually increased the survival time of the embryo.

(ii) Adverse effect of chorion on survival

When survival times of ruptured and entire embryos were compared statistically, the value of P was 0.1 in Experiment 1 and 0.2 in Experiment 2. The standard deviations were 31 hours and 21 hours respectively. The differences between ruptured and entire embryos were not considered to be significant in either experiment. Consequently, Experiment 3 was designed to compare the survival of much larger samples of embryos, which would be observed more frequently.

In Experiment 3, ruptured 15 and 25-hour-old embryos survived about 10 hours longer than entire embryos, so that the two curves in Fig. 12 are roughly parallel. This suggests that the effect of rupturing (in prolonging survival time) may be independent of age. When survival times of ruptured and entire embryos in Experiment 3 were compared, the value of P was less than 0.001 (Table 7), and the standard deviation 16 hours. When the standard deviations of ruptured and entire embryos were estimated separately, the values were 20 and 10 hours, respectively. The greater variability of the ruptured embryos was probably owing to variations in the

extent of the rupturing. The difference in survival between ruptured and entire embryos was considered to be highly significant. To account for this surprising result, the following explanation is offered.

The first sign that an entire embryo was dying from zinc poisoning was the formation of opaque patches of material, within the perivitelline fluid which surrounds the embryo (Fig. 13). The patches gradually increased in number and size until they coalesced to form a 'cocoon', in the centre of which the living embryo could still be seen (Fig. 14). Later, the tissues of the embryo itself turned opaque until finally the animal became immobilized. An unaffected embryo is illustrated in Fig. 15.

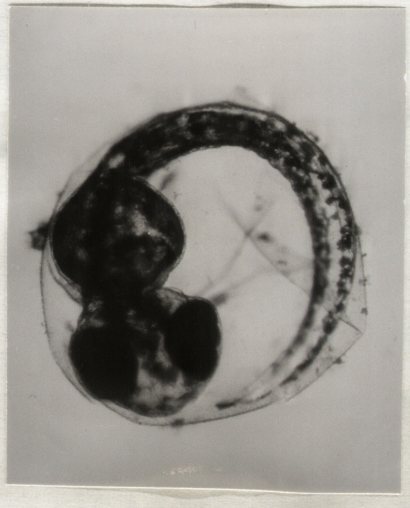
A naked or ruptured embryo, which was exposed to a toxic solution of zinc, did not become enclosed in a mass of opaque material because the perivitelline fluid had previously become dispersed in the external environment. The embryo did however turn opaque in a similar manner to an entire embryo. Comparable changes developed appreciably later in naked and ruptured embryos.

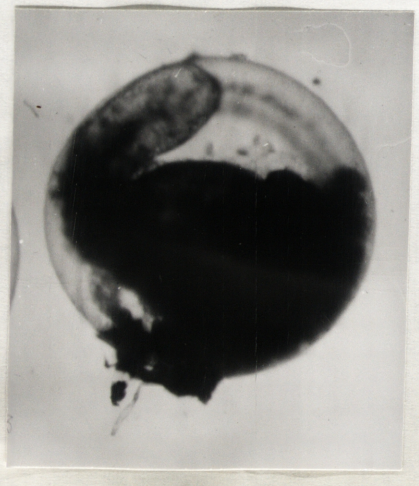
It is suspected that entire embryos became immobilized earlier than naked embryos, in similar concentrations of zinc, because some vital metabolic process (such as gas exchange) was slowed down by the combined obstruction of the opaque

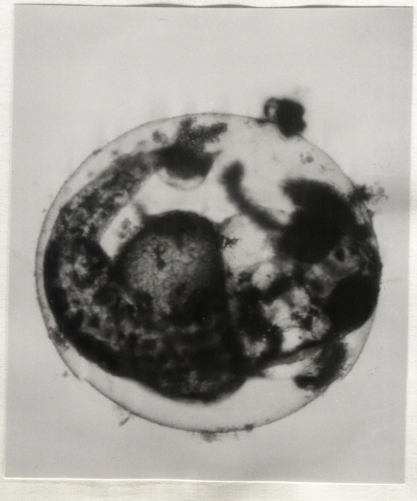
Fig. 13. 64-hour-old zebrafish embryo with chorion entire, exposed to 40 p.p.m. zinc for 34 hours. Opaque material forming inside chorion and plugging micropyle. (x40)

Fig. 14. 53-hour-old zebrafish with chorion entire, exposed to 40 p.p.m. zinc for 50 hours. 'Cocoon' of opaque material surrounding head and yolk of embryo. Micropyle plugged. (x40)

Fig. 15. 64-hour-old zebrafish embryo with entire chorion. Chorion intact but dented by handling. Embryo reared in tap water. (x40)







material plus the chorion. It would be difficult to prove this supposition, but it has been demonstrated by Hayes, Wilmot and Livingstone (1951) that in well aerated water newly hatched salmon embryos take up oxygen 1.5 times faster than embryos about to hatch. It is also significant that ruptured zebrafish embryos were more active than entire embryos under similar conditions. This difference in activity was demonstrated in the following manner.

The rates of body movements of ruptured and entire 30-hour-old embryos were compared, both in tap water and zinc sulphate solution (20 p.p.m. Zn) in tap water. At the time that the observations were made, no opaque material was present either in the perivitelline fluid or in the embryos. The chorion of the treated embryos had been ruptured at 13 hours. Half the ruptured embryos and half the entire embryos had been transferred to the zinc solution at 15 hours, the remainder being maintained in tap water. Four observations were made on each of the four groups. The times required for subsamples of five embryos to make twenty-five body movements were recorded, and rates of body movements per embryo per minute calculated.

The average rates of activity of ruptured and entire embryos in tap water were 13.3 and 6.9 movements per embryo per minute respectively. Comparable rates in zinc solution

were 13.2 and 6.5 movements, respectively. When the activity of ruptured and entire embryos was compared statistically, the value of P was less than 0.001. When the activity of embryos held in zinc solution was compared with that of those held in tap water, the value of P was 0.2. The standard deviation of the observations was 3.0 movements per embryo per minute. It is concluded from these data that the difference in activity between ruptured and entire embryos was highly significant, but that no difference in activity was caused by the different solutions. It is expected that the difference in activity between ruptured and entire embryos would have been even greater once opaque material began to form in the perivitelline fluid.

(iii) Function of the chorion

The discovery that the chorion may be a liability to the zebrafish embryo, by actually reducing its survival during exposure to zinc poisoning, leads to speculation concerning the value of this membrane to the developing embryo. It is difficult to see how the intact chorion can be of much advantage to the embryo in tap water, because naked and ruptured embryos develop normally from the blastula stage onwards. Their survival is at least as good as the survival of entire embryos.

Microscopic examination reveals that the chorion is unlikely to function as a semipermeable membrance. It appears like a sieve under high magnification, being completely perforated by numerous radial canals. The diameter of the canals was estimated to be approximately 1.3 $\mu$ when measured both by using a Cooke A.E.I. image - splitting eye piece and by examining photomicrographs prepared under normal and phase-contrast illumination (Figs. 16 & 17). The prominent rings in Fig.16 are interpreted to be shadows cast within the canal walls.

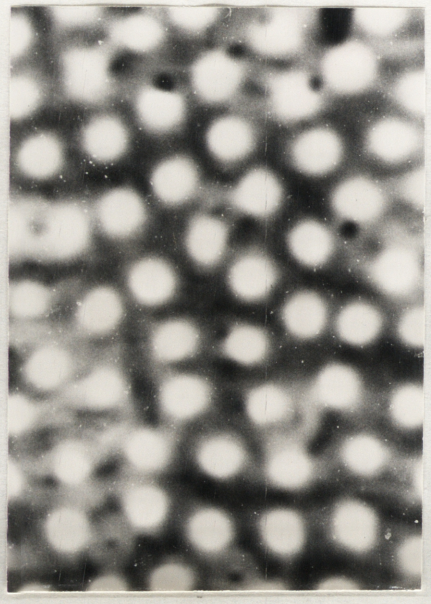
In addition to the radial canals, the chorion is broken by a large pore - the micropyle (Fig. 18) - which may be up to 100 $\mu$ across and may remain open until hatching. The combined area of all the radial canals is estimated to be about 39%, and the area of the micropyle about 1%, of the total surface of the chorion. Colonies of Paramecium were occasionally seen in the perivitelline fluid of entire embryos, presumably having entered the egg through the micropyle. It may be predicted from the foregoing evidence that only the largest molecules in the perivitelline fluid would be retained by such a discontinuous barrier as the chorion, especially once the embryo began to move about.

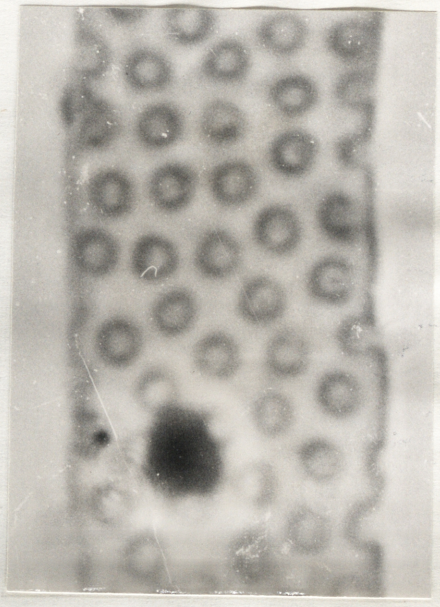
Staining with vital dyes supports the prediction. Both nile blue and janus green penetrated the unruptured chorion

Fig. 16. Chorion of 30-hour-old zebrafish embryo, showing radial canals through membrane, under normal illumination. Fixed in Bouin's solution, sectioned at 9 μ. and stained with eosin. Surface view. (x5000)

Fig. 17. Chorion of 30-hour-old zebrafish embryo, showing radial canals through membrane, under phase-contrast illumination. Fresh material, mounted in water. (x5000)

Fig. 18. 31-hour-old zebrafish embryo with chorion entire, showing micropyle. Some perivitelline fluid has been forced out of micropyle by handling. (x40)





of developing zebrafish eggs and stained both the embryo and particles in the perivitelline fluid within 1 minute. Eosin in 70% alcohol also penetrated the chorion rapidly, killing the embryo and staining it. A careful examination of fragments of chorion stained with eosin failed to reveal any trace of material plugging the radial canals. It is concluded from all the above data that the chorion of the zebrafish egg is permeable to many small particles.

The chorion of most teleost eggs is believed to be largely impermeable to salts, although in some eggs diffusion through the chorion has been definitely established. Becher (1928) observed numerous radial canals through the chorion of trout eggs, similar in diameter (1.4 $\mu$) to those in zebrafish eggs. The mechanisms of osmoregulation in unhatched teleost eggs may therefore vary widely, and this alone would justify further study. It would also be interesting to learn how the resistance to poisons of other teleost eggs compares with the resistance of hatched fish of the same species.

Returning to the chorion of the zebrafish egg, it is suggested that its normal function is not to interfere with the exchange of ions and molecules between the perivitelline fluid and the external environment, but rather to protect the embryo from small predators and mechanical damage. Copepods and ostracods have been observed to graze on inactive, newly

hatched fish and to kill them, although the predators were unable to penetrate through the chorion of unhatched embryos.

## 5. RELATION BETWEEN OXYGEN UPTAKE AND RESISTANCE

### a) Materials and methods

#### (i) Rearing conditions

Zebrafish of common parentage were bred and reared in Canberra tap water, total hardness 10 p.p.m. $CaCO_3$, temperature 25°C., pH between 6.8 and 7.2, and dissolved oxygen concentration at least 6 p.p.m., under conditions described in Section 3a. Test fish aged 13 days or more were starved for 24 hours before oxygen consumption was measured. Younger fish were not fed at all. Mortality was similar to that described in Section 3a.

#### (ii) Measurement of survival time in zinc

Data on resistance to zinc poisoning were derived from the toxicity bioassays described in Section 3 and summarized in Fig. 5.

#### (iii) Measurement of oxygen uptake

A Warburg constant-volume respirometer was used to measure the oxygen consumption in tap water of samples of

fish aged 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, 40, and 100 days (Umbreit, Burris & Stauffer, 1957). All determinations were made at a temperature of 25.0 ± 0.1°C., total hardness initially 10.0 ± 0.5 p.p.m. $CaCO_3$, pH between 6.8 and 7.2 and dissolved oxygen concentration initially 8 p.p.m. A sample of fish of known age was transferred to a small volume of water in a Warburg flask, and 0.2 ml. of 10% potassium hydroxide solution and a wick of filter paper added to the well of the flask. The flask was wrapped in aluminium foil to reduce sensory stimulation of the fish. After assembly, the apparatus was left for several hours before readings were made, so that the oxygen consumption of the fish could become stabilized. Except with 13-day-old fish, measurements were recorded for 17 hours or until 100 µl. oxygen had been used. The recording period for 13-day-old fish was terminated at 5 hours because mortality was higher. With all age groups, observations were discarded if any fish died. Seventy-three valid determinations were made, involving 1223 fish. Variable features of the experimental procedure are summarized in Table 8.

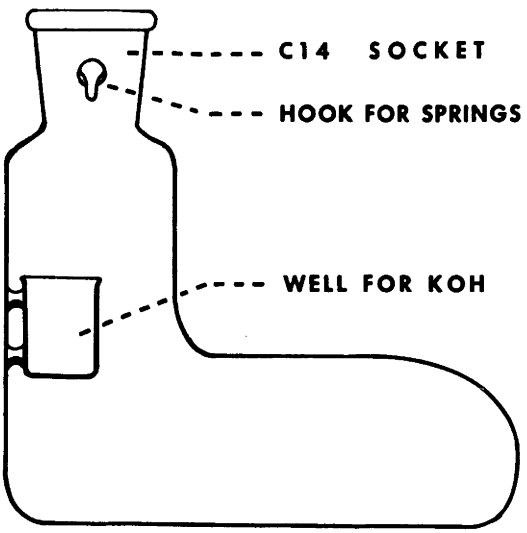
During the determinations, oxygen concentration would be expected to fall slightly, as oxygen in the system was used up. As never more than 200 µl. of oxygen were consumed in any determination, out of an air volume of at least 11,000 µl.

Table 8. Variable features of the procedure used to measure oxygen consumption.

| Age of fish | Sample size | Number of samples | Volume of flask | Volume of water | Stabiliz-ation period | Recording period |
|---|---|---|---|---|---|---|
| 0 days | 25 | 4 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 1 day | 25 | 6 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 2 days | 25 | 7 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 3 days | 25 | 7 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 4 days | 25 | 5 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 5 days | 25 | 3 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 6 days | 25 | 5 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 7 days | 25 | 2 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 8 days | 25 | 4 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 10 days | 25 | 3 | 14 ml. | 2.8 ml. | 4 hr. | 17 hr. |
| 13 days | 25 | 2 | 14 ml. | 2.8 ml. | 4 hr. | 5 hr. |
| 40 days | 1 | 10 | 14 ml. | 2.8 ml. | 4 hr. | $<8$ hr. |
| 100 days | 1 | 13 | 35 ml.* | 15 ml. | 2 hr. | $<2$ hr. |

* Specially designed flask illustrated in Fig. 19.

Fig. 19. Drawing of flask used for measuring oxygen consumption of adult zebrafish. Actual size. Capacity of flask, 35 ml. Capacity of well, 0.7 ml. Flask attached directly to standard Warburg manometer by a size C14 'Quickfit' socket.


C14 SOCKET
HOOK FOR SPRINGS
WELL FOR KOH

it can be calculated that the dissolved oxygen concentration never fell below 7.3 p.p.m.

(iv) Relative rate of oxygen uptake

The absolute rate of oxygen uptake could be expressed as the volume of oxygen consumed per sample of fish per unit time (e.g. $\mu$l. $O_2$/ 25 fish / hour). For further analysis, the relative rate of oxygen uptake was calculated as the weight of oxygen consumed per unit weight of fish per unit time (mg. $O_2$/ kg. fish / hour).

No attempt was made to determine the precise weights of samples of 25 eggs or newly hatched fish used in the respiration experiments, because the fish were too small. The average weight of samples of fish aged 11 days or less was determined in the following manner. A 6-ml. plastic vial was dried in a vacuum dessicator for 24 hours at 25°C. Immediately the dessicator was opened, the vial was capped and allowed to stand for 30 minutes before being weighed. A counted number of fish (approximately 200) in a small volume of water was added to the vial and free water removed by Pasteur pipette and filter paper. The wet weight of the fish was determined and the sample dried to constant weight in the vacuum dessicator, using the same weighing procedure as before. All together, 1904 fish aged 11 days or less were

weighed in eight samples. The average weight of each age class up to 13 days was estimated by graphical analysis. Wet and dry weights of 40 and 100-day-old fish were obtained by weighing the actual fish used in the respiration experiments, using a similar procedure to that with smaller fish.

b) Results

(i) Absolute rate of oxygen uptake

The absolute rate of oxygen uptake, of samples of twenty-five zebrafish aged from 0 to 13 days, is shown in Fig. 20, the solid line being fitted to the mean values for different age groups. Oxygen uptake increased to a maximum of 5.8 µl. / 25 fish / hour on the fifth day and then declined. The oxygen uptake of individual 40 and 100-day-old zebrafish is plotted in Fig. 21. The mean values for the two age groups were 14 and 90 µl. / 1 fish / hour, respectively. (The mean oxygen uptake of individual zebrafish aged 0 to 13 days is also plotted in Fig. 21, from data in Fig. 20.)

(ii) Weight of fish

Mean wet and dry weights of samples of zebrafish aged from 0 to 100 days are listed in Table 9 and the data for fish aged 11 days or less are plotted in Fig. 22.

Fig. 20. Oxygen consumption in tap water of zebrafish aged 13 days or less (solid line). Each point indicates data for twenty-five fish. Survival curve of fish of approximately similar ages in 40 p.p.m. zinc (broken line), redrawn from Fig. 5.

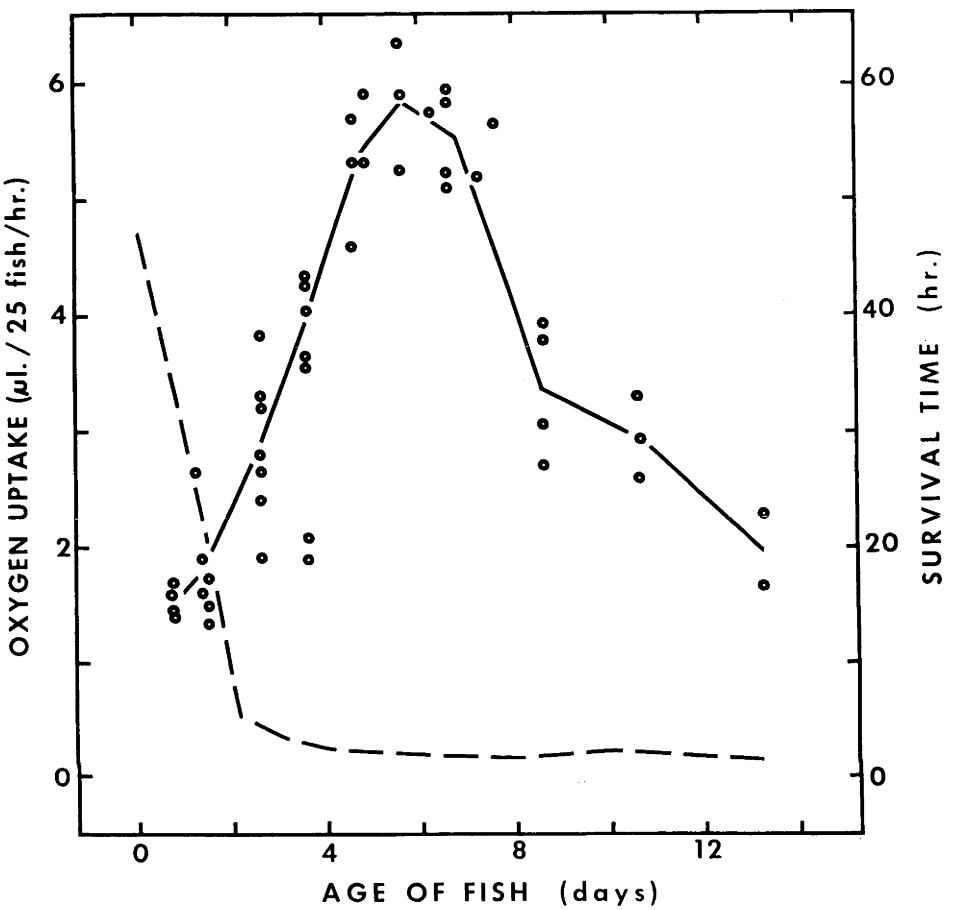

OXYGEN UPTAKE (µl./25 fish/hr.)
SURVIVAL TIME (hr.)
AGE OF FISH (days)
0
2
4
6
0
20
40
60
0
4
8
12

Fig. 21. Mean oxygen uptake of individual zebrafish plotted against dry weight, both on logarithmic scales. Line fitted by eye to data for thirteen fish aged 100 days (weight 43 to 100 mg.), ten fish aged 40 days (weight 1.7 to 13 mg.), and 1200 fish aged from 0 to 13 days (weight 0.049 to 0.016 mg.). Data for oxygen uptake of fish aged from 0 to 13 days taken from Fig. 20. Estimates of weight of fish aged 13 days or less based on Curve C of Fig. 22 (heavy circles) and Curve B (light circles). Regression line fitted to heavy circles, excluding data for fish aged 0 to 4 days.

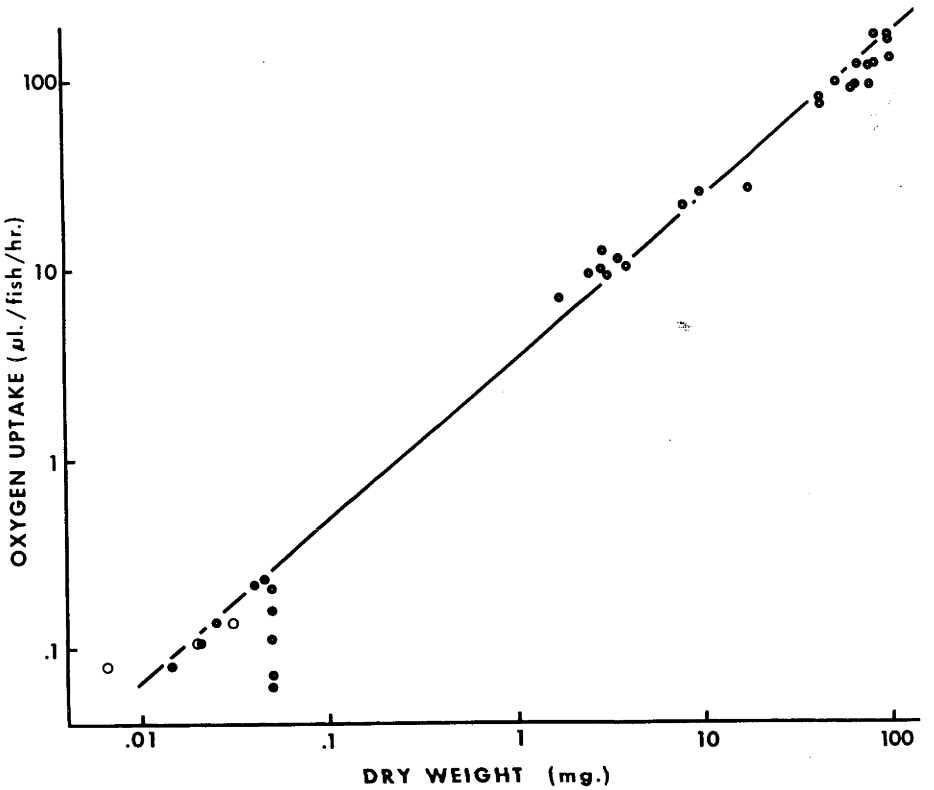
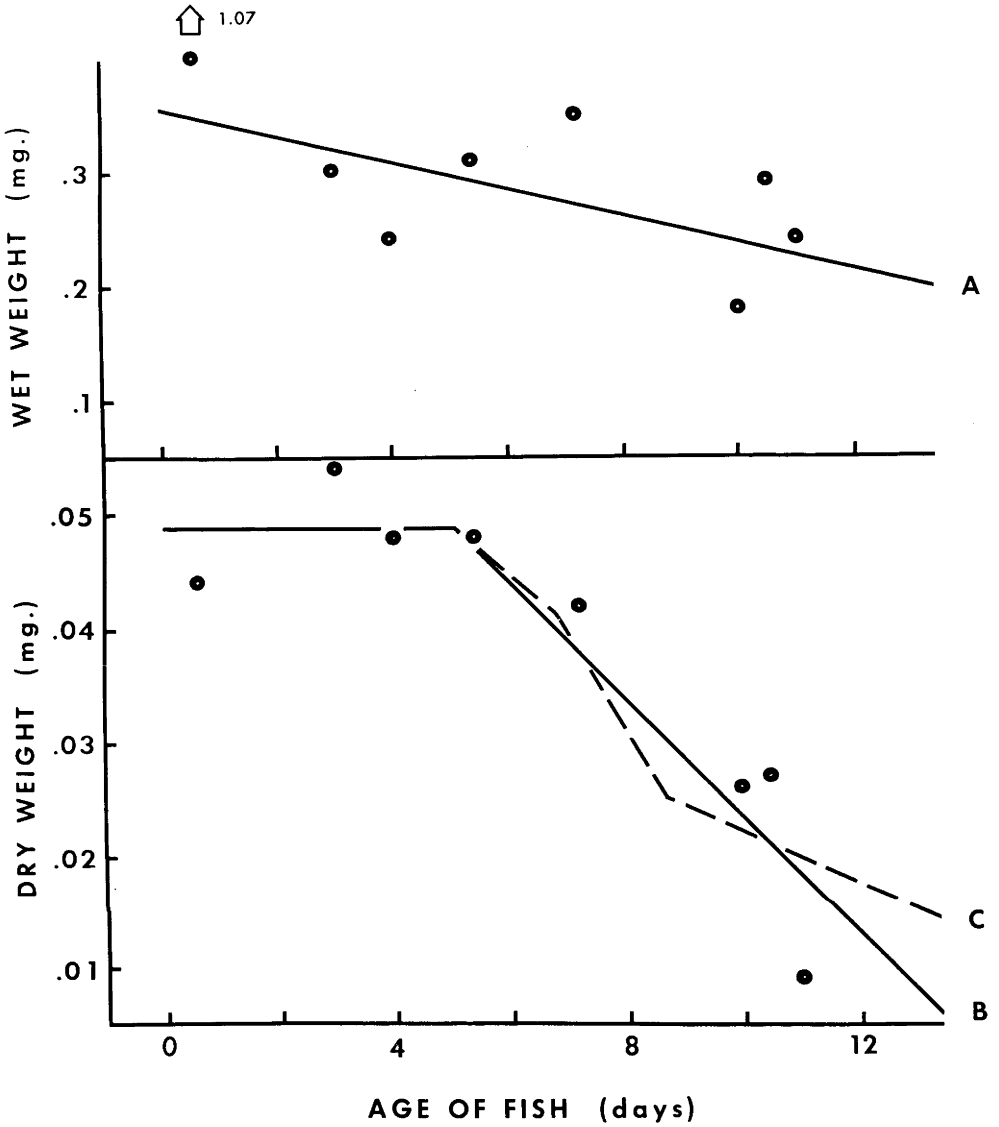

OXYGEN UPTAKE (µl./fish/hr.)
100
10
1
.1
.01
.1
1
10
100
DRY WEIGHT (mg.)

Table 9. Mean wet and dry weights of samples of zebrafish aged from 0 to 100 days.

| Age of fish | Sample size | Wet weight per fish | Dry weight per fish |
|---|---|---|---|
| 0.6 days | 449 | 1.07 mg. | 0.044 mg. |
| 3.0 days | 283 | 0.30 mg. | 0.054 mg. |
| 4.0 days | 213 | 0.24 mg. | 0.048 mg. |
| 5.4 days | 341 | 0.32 mg. | 0.048 mg. |
| 7.2 days | 132 | 0.35 mg. | 0.042 mg. |
| 10.0 days | 183 | 0.18 mg. | 0.026 mg. |
| 10.5 days | 201 | 0.28 mg. | 0.027 mg. |
| 11.0 days | 102 | 0.24 mg. | 0.009 mg. |
| 40±5 days | 10 | 26.1 mg. | 5.1 mg. |
| approx. 100 days | 13 | 324 mg. | 74.2 mg. |

Fig. 22. Mean wet and dry weights of samples of zebrafish aged 0 to 11 days. Data from Table 9. Line A, estimated wet weights assuming linear reduction in weight. Line B, estimated dry weights assuming constant weight until fifth day and linear reduction in weight from fifth to thirteenth day. Lines A and B fitted by eye. Line C fitted to dry weights calculated from data in Fig. 24.


1.07
WET WEIGHT (mg.)
.3
.2
.1
A
.05
DRY WEIGHT (mg.)
.04
.03
.02
.01
C
B
0
4
8
12
AGE OF FISH (days)

The large reduction in wet weight (Fig. 22) from the first to the third day is assumed to have resulted from hatching at about the third day, because dry weight remained relatively unchanged. Reduction in wet weight of unhatched embryos and newly hatched fish is assumed to have progressed in equal daily increments of 3.3% of the initial weight, from the first to the thirteenth day (Line A). Line A roughly fits the observed data. Dry weight (Fig. 22) is assumed to have remained constant until the fifth day (while oxygen uptake was slow), and then to have decreased by equal daily increments of 10.5% of the initial weight, from the sixth until the thirteenth day (while the rate of oxygen uptake is believed to have been rapid). The resulting line (B) roughly fits the observed data. Lines A and B were fitted by eye.

The dry weights of the 40 and 100-day-old zebrafish used in respiration measurements are indicated in Fig. 21.

(iii) Relative rates of oxygen uptake

Figure 23 (solid line) gives the same oxygen data as Figs. 20 and 21, recalculated to express the mean oxygen uptake of each age group of test animals as mg. oxygen per kg. fish (wet weight) per hour. Estimates of the wet weights of fish aged 13 days or less were read from Line A of Fig. 22.

Fig. 23. Oxygen uptake of zebrafish aged 0 to 100 days (solid line), calculated per unit wet weight of fish. Data from Fig. 20 (solid line) and Fig. 22 (Line A). Survival curve of fish of approximately similar ages in 40 p.p.m. zinc (broken line), redrawn from Fig. 5.

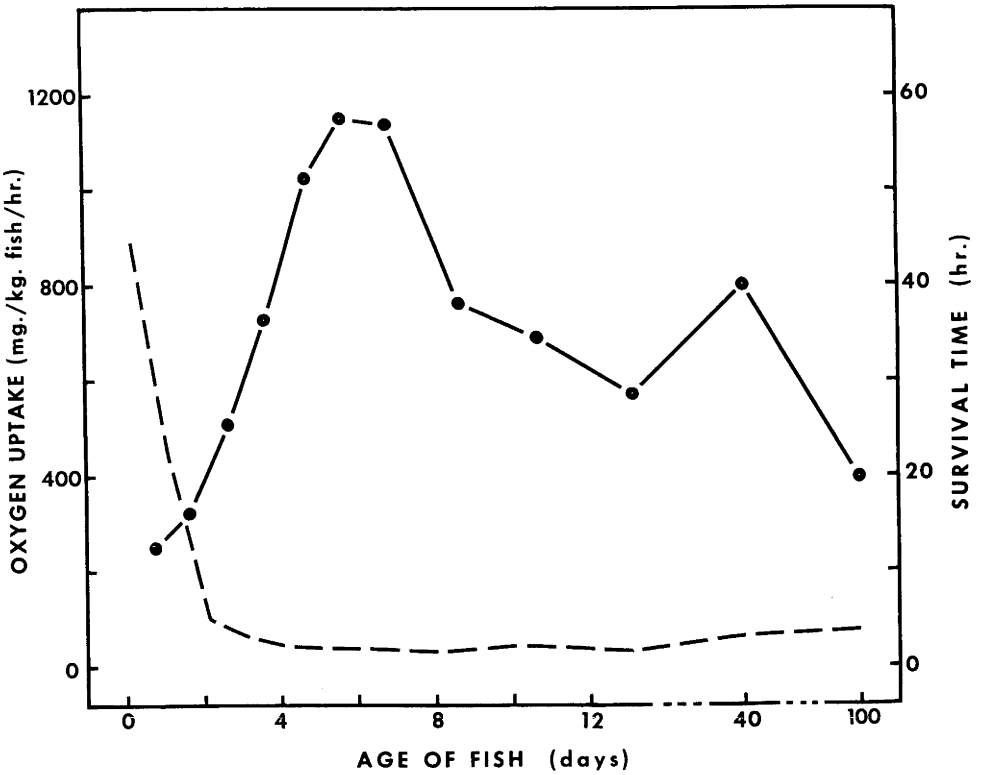

1200
800
400
0
OXYGEN UPTAKE (mg./kg. fish/hr.)
60
40
20
0
SURVIVAL TIME (hr.)
0
4
8
12
40
100
AGE OF FISH (days)

Figure 24 (solid line) shows the same data as Fig. 23, this time with oxygen uptake calculated per kg. fish (dry weight), for zebrafish of identical ages to those for which survival data are available. (See Section 5b (iv).) Estimates of dry weight of fish aged 13 days or less were read from Line B of Fig. 22.

(iv) Survival time in zinc

All survival data which will be discussed in Section 5c are plotted in Fig. 5. Survival data in 40 p.p.m. zinc solution are indicated in Figs. 20, 23 and 24 by means of broken lines.

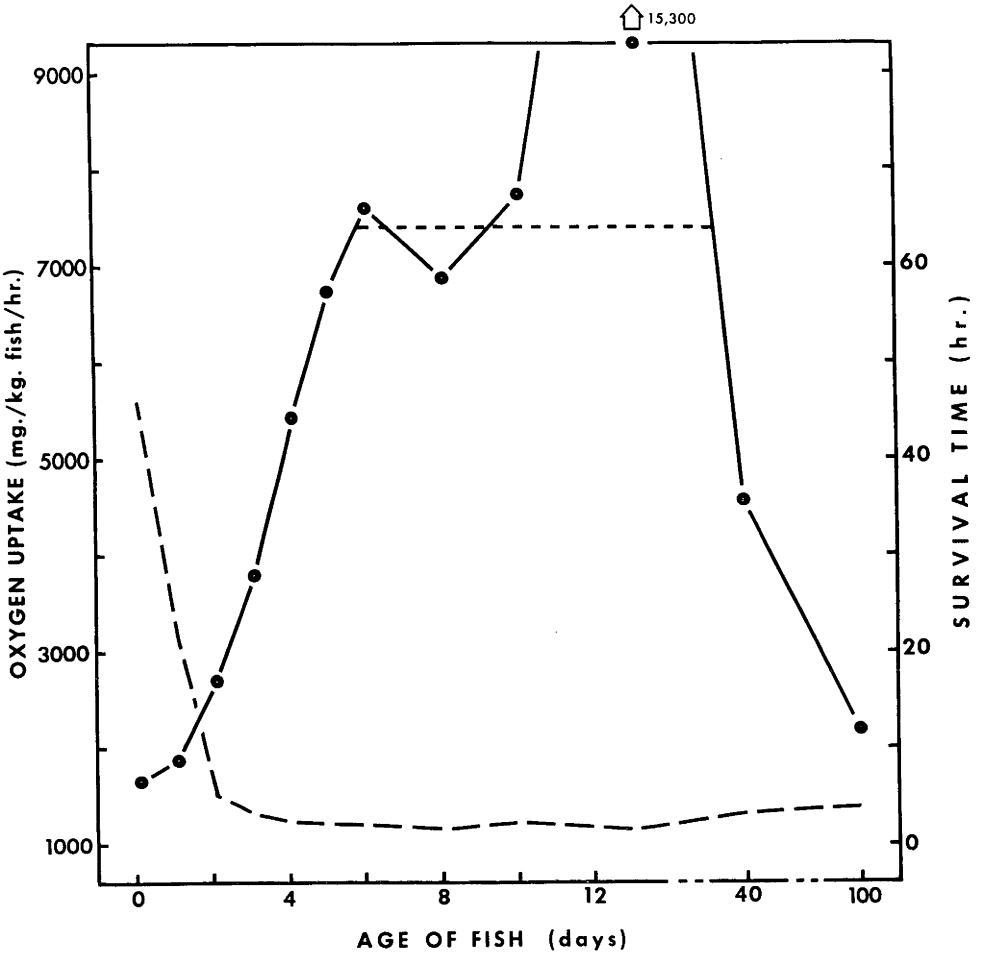
c) Discussion

The purpose of this section is to consider Hypothesis 2 of Section 3c, which is that the resistance of the zebrafish to zinc sulphate is inversely proportional to the rate of oxygen uptake of the fish.

(i) Resistance to zinc and absolute rate of oxygen uptake

A comparison of the two curves in Fig. 20 indicates that oxygen uptake increased with age until the fifth day and then declined until the thirteenth, whereas survival time in 40 p.p.m. zinc solution decreased with age until the fourth day

Fig. 24. Oxygen uptake of zebrafish aged 0 to 100 days (solid line), calculated per unit dry weight of fish. Data from Fig. 20 (solid line) and Fig. 22 (Line B). Survival curve of fish of identical ages in 40 p.p.m. zinc (broken line), redrawn from Fig. 5.

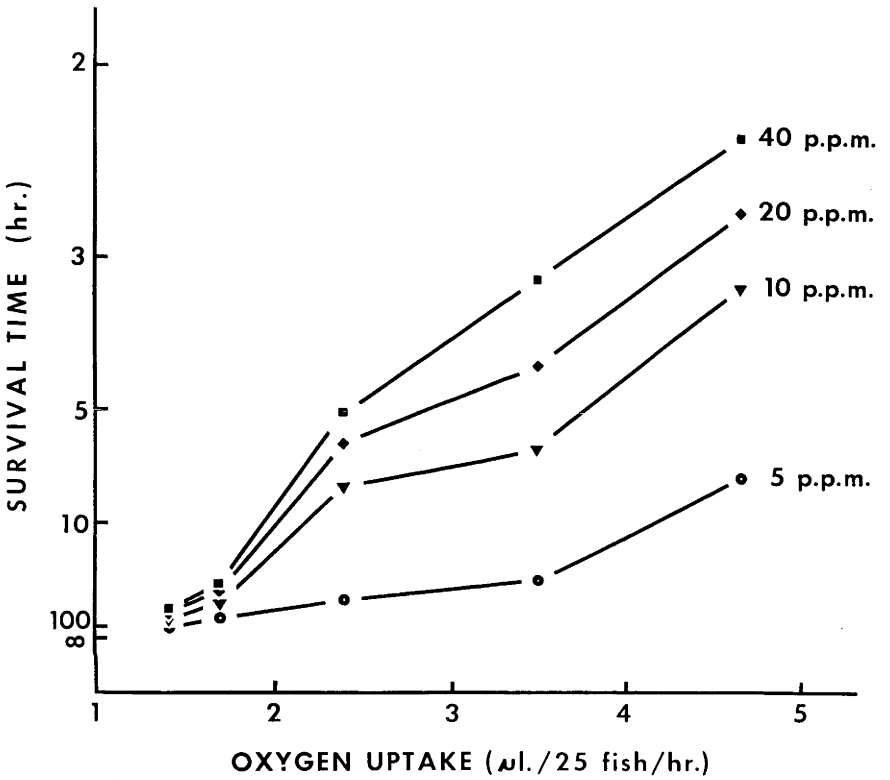

15,300
9000
7000
5000
3000
1000
OXYGEN UPTAKE (mg./kg. fish/hr.)
60
40
20
0
SURVIVAL TIME (hr.)
0
4
8
12
40
100
AGE OF FISH (days)

and then remained fairly constant. (Survival time in a given concentration of zinc is taken to be a measure of resistance.) The survival time of adult fish was slightly longer than that of newly hatched fish (Fig. 5), but their oxygen uptake was naturally much higher (Fig. 21). Survival times in 20, 10 and 5 p.p.m. zinc (Fig. 5) usually followed the same trend as survival time in 40 p.p.m. zinc. There would appear to be an inverse correlation between oxygen uptake and survival time until the fourth day. The possibility is tested graphically in Fig. 25, by plotting survival time on a reciprocal scale against oxygen uptake of fish of identical age. It is concluded that the two variables as plotted were directly correlated, and consequently that oxygen uptake and survival time were inversely correlated. The second hypothesis is therefore supported by data for fish up to 4 days old, but apparently not by data for older fish.

(ii) Resistance and relative rate of oxygen uptake

Comparison of the two curves in Fig. 23 indicates that survival time in 40 p.p.m. zinc and oxygen uptake calculated on a wet weight basis were inversely correlated until the fourth day. Comparison of Figs. 5 and 23 suggests the same conclusion concerning all four test concentrations of zinc.

Fig. 25. Survival time measured on a reciprocal scale of zebrafish in four concentrations of zinc, plotted against oxygen uptake of fish of identical age. Reading from left to right, ages of fish were 0.1, 1.1, 2.1, 3.1, and 4.1 days. Data from Figs. 5 and 20, respectively.


SURVIVAL TIME (hr.)
2
3
5
10
100
80
1
2
3
4
5
OXYGEN UPTAKE (µl./25 fish/hr.)
40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.

The second hypothesis is again supported by data for fish up to 4 days old.

When oxygen uptake was calculated per unit dry weight of fish (Fig. 24), it increased steadily until the fifth day and then fluctuated about a mean value of 7400 mg./kg. fish/hour from the sixth to the tenth day (dotted line). The oxygen uptake of 40 and 100-day-old fish was lower, but the uptake of 13-day-old fish was extremely high. This last result is considered to be unreliable because 13-day-old fish were inactive and could reasonably be expected to useless than 7400 mg. oxygen/kg. fish/hour. The high result probably arose from an underestimate of the dry weight of 13-day-old fish (Section 5b (ii)). All the data for fish aged from 6 to 13 days would lie along the dotted line in Fig. 24, had Line C of Fig. 22 been used to estimate dry weight, instead of Line B.

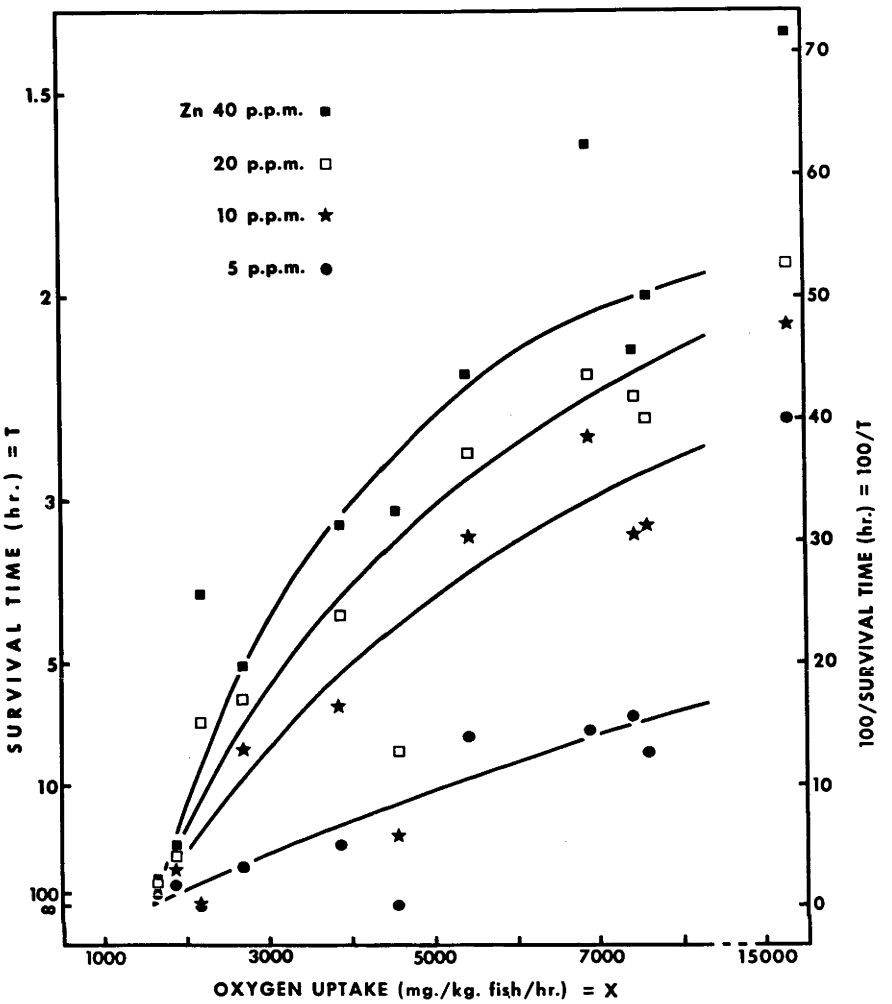
In Fig. 21, the dry weight of fish aged from 6 to 13 days has been estimated both from Line C (heavy circles) and Line B (light circles). The regression line was fitted to the heavy circles. However, in the discussion which follows, Line B will be used to calculate the dry weight of young fish, unless otherwise stated.

Comparison of the solid and broken lines in Fig. 24 indicates that survival time in 40 p.p.m. zinc was inversely

correlated with oxygen uptake throughout the life cycle, with the apparent exception of data for 13-day-old fish. A similar correlation appeared to exist between oxygen uptake and most survival data for 20, 10 and 5 p.p.m. zinc. The possibility is tested graphically in Fig. 26, as in Fig. 25, by plotting survival time on a reciprocal scale against the oxygen uptake of fish of identical age. To facilitate further calculation, the reciprocals of survival time (multiplied by 100 to eliminate fractions) are also shown in Fig. 26. Most of the data may be fitted to four curvilinear regression lines, with the following exceptions.

Between 10 and 50% of the adult and juvenile fish which were exposed to 5 or 10 p.p.m. zinc died at about the time at which they would be expected to die, were the data to fit the appropriate regression line. The remainder survived indefinitely or for an extended period. However, the survivors were observed to suffer acute respiratory distress at their predicted time of immobilization. Thus all the fish responded in some way at the expected time. It is suggested that the toxic action of the zinc was counterbalanced in the survivors by the successful reaction of the fish, following the sequence of events proposed by Lloyd (1962) and described in Section 2c (v). This point will be considered further in Section 7c (i). The survival data for 13-day-old fish may be

Fig. 26. Survival time (T) on a reciprocal scale, of zebrafish in four concentrations of zinc, plotted against the oxygen uptake per unit dry weight of fish (X) for fish of identical age. Reciprocals of survival time multiplied by 100 (100/T) are indicated. Curves calculated from Equation 5 and Table 10. Observed data marked by symbols, from Figs. 5 and 24.


Zn 40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.
SURVIVAL TIME (hr.) = T
100/SURVIVAL TIME (hr.) = 100/T
OXYGEN UPTAKE (mg./kg. fish/hr.) = X
1.5
2
3
5
10
100
70
60
50
40
30
20
10
0
1000
3000
5000
7000
15000

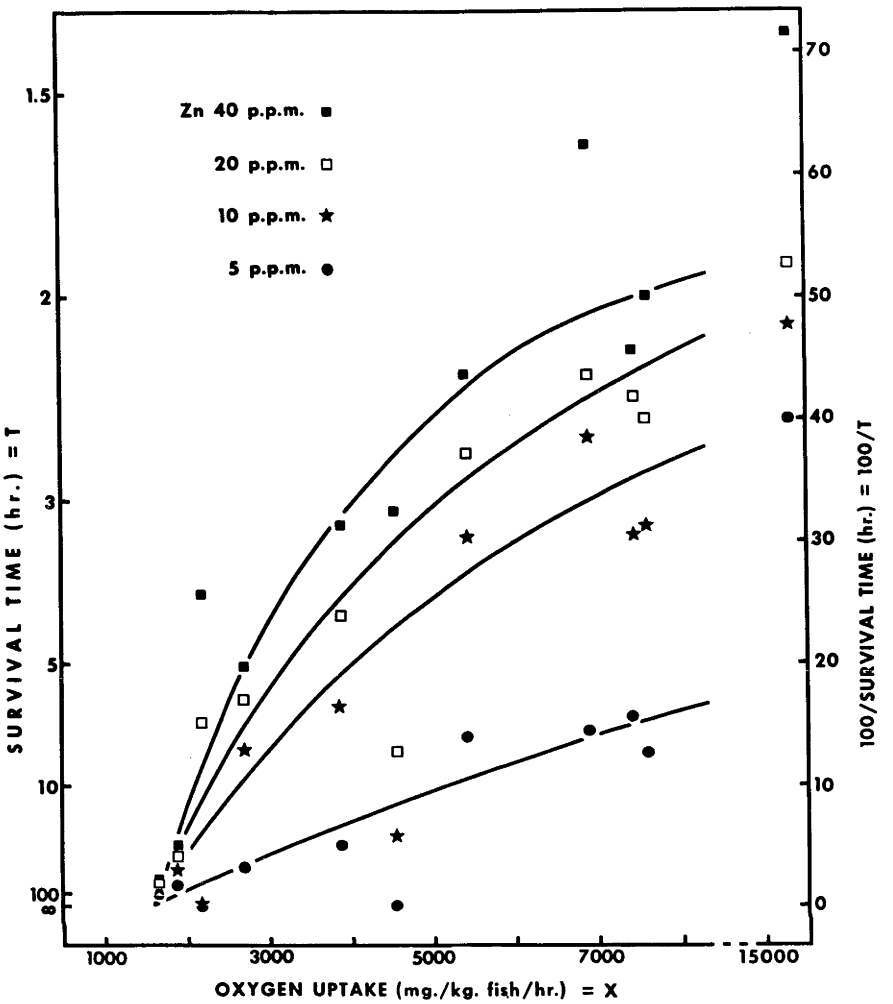
misleading because the expectation of life of the test fish was only a few hours. The oxygen uptake data for 13-day-old fish have been shown to be unreliable (Section 5c (ii), paragraph 2). Most of the scatter in the data for fish aged 10 days or less was owing to minor fluctuations in survival time (Fig. 5), greatly magnified by taking reciprocals.

In the discussion which follows, the survival time of zebrafish in hours, when exposed to a given concentration of zinc, will be symbolized by T. The reciprocal of survival time multiplied by 100 therefore becomes $\frac{100}{T}$. The relative rate of oxygen uptake in mg. per kg. fish (dry weight) per hour will be symbolized by X.

The relation between rate of oxygen uptake (X), and the reciprocal of survival time in zinc ($\frac{100}{T}$), has been shown to be curvilinear (Fig. 26). Hypothesis 2 (that resistance is inversely proportional to oxygen uptake) must therefore be rejected in its original form. The shape of the four curves in Fig. 26 indicates that as X increased from a threshold value $X_S$ when $\frac{100}{T} = 0$, the rate of increase of $\frac{100}{T}$ decreased. This means that the fish survived longer than would be predicted, were the relationship linear and the original hypothesis correc

A simple explanation of this result could be that T consists of two components; a threshold survival time ($T_S$) which is independent of X, and a reaction time ($T-T_S$) which is inversely proportional to X. Hypothesis 2a is therefore propose

Fig. 26. Survival time (T) on a reciprocal scale, of zebrafish in four concentrations of zinc, plotted against the oxygen uptake per unit dry weight of fish (X) for fish of identical age. Reciprocals of survival time multiplied by 100 (100/T) are indicated. Curves calculated from Equation 5 and Table 10. Observed data marked by symbols, from Figs. 5 and 24.


Zn 40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.
SURVIVAL TIME (hr.) = T
100/SURVIVAL TIME (hr.) = 100/T
OXYGEN UPTAKE (mg./kg. fish/hr.) = X
1.5
2
3
5
10
100
70
60
50
40
30
20
10
0
1000
3000
5000
7000
15000

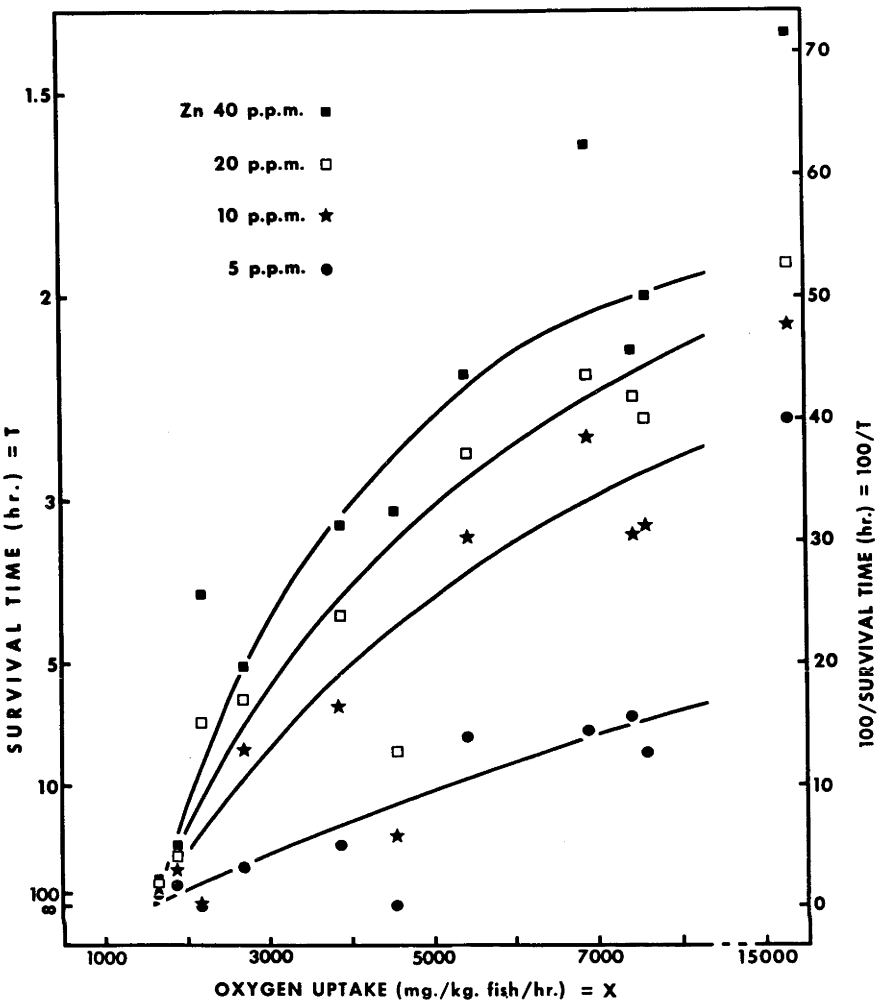
misleading because the expectation of life of the test fish was only a few hours. The oxygen uptake data for 13-day-old fish have been shown to be unreliable (Section 5c (ii), paragraph 2). Most of the scatter in the data for fish aged 10 days or less was owing to minor fluctuations in survival time (Fig. 5), greatly magnified by taking reciprocals.

In the discussion which follows, the survival time of zebrafish in hours, when exposed to a given concentration of zinc, will be symbolized by T. The reciprocal of survival time multiplied by 100 therefore becomes $\frac{100}{T}$. The relative rate of oxygen uptake in mg. per kg. fish (dry weight) per hour will be symbolized by X.

The relation between rate of oxygen uptake (X), and the reciprocal of survival time in zinc ($\frac{100}{T}$), has been shown to be curvilinear (Fig. 26). Hypothesis 2 (that resistance is inversely proportional to oxygen uptake) must therefore be rejected in its original form. The shape of the four curves in Fig. 26 indicates that as X increased from a threshold value $X_S$ when $\frac{100}{T} = 0$, the rate of increase of $\frac{100}{T}$ decreased. This means that the fish survived longer than would be predicted, were the relationship linear and the original hypothesis correct

A simple explanation of this result could be that T consists of two components; a threshold survival time ($T_S$) which is independent of X, and a reaction time ($T-T_S$) which is inversely proportional to X. Hypothesis 2a is therefore proposed

Fig. 26. Survival time (T) on a reciprocal scale, of zebrafish in four concentrations of zinc, plotted against the oxygen uptake per unit dry weight of fish (X) for fish of identical age. Reciprocals of survival time multiplied by 100 (100/T) are indicated. Curves calculated from Equation 5 and Table 10. Observed data marked by symbols, from Figs. 5 and 24.


Zn 40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.
SURVIVAL TIME (hr.) = T
100/SURVIVAL TIME (hr.) = 100/T
OXYGEN UPTAKE (mg./kg. fish/hr.) = X
1.5
2
3
5
10
100
70
60
50
40
30
20
10
0
1000
3000
5000
7000
15000

that the reaction time of zebrafish to zinc poisoning, in a given concentration of zinc, is inversely proportional to oxygen uptake. This possibility may be tested graphically by plotting the reciprocal of reaction time ($\frac{100}{T-T_S}$) against X, for different values of $T_S$. If a linear relationship can be found for a particular value of $T_S$, the hypothesis is supported. This was in fact found to be the case when $T_S$ = 1.3 hours (Fig. 27). The four linear regression lines were fitted by eye. The equation of the four lines is

$$\frac{100}{T-T_S} = m(X-X_S)^n \qquad (4)$$

where m is the slope. Since the lines were assumed to be straight, n must equal unity. Substituting $\frac{100}{m} = K$, and cross-multiplying, the equation may be rewritten

$$(X-X_S)^n(T-T_S) = K \qquad (5)$$

The values for $X_S$, $T_S$, n and K are listed in Table 10 for the four concentrations of zinc.

It is concluded from Fig. 27 that the relationship between $\frac{100}{T-T_S}$ and X is linear. Hypothesis 2a (that reaction time is inversely proportional to oxygen uptake) is therefore supported. If this relationship should prove to be a general one, with other fish and other poisons, any factor which alters the rate of oxygen uptake - such as temperature, activity, stress, growth or starvation - may be predicted to modify the survival time of fish in poison. Hypothesis 2a may therefore prove to be of central importance in problems of fish toxicology.

Fig. 27. Reaction time ($T-T_S$) on a reciprocal scale, of zebrafish in four concentrations of zinc, plotted against the oxygen uptake per unit dry weight of fish (X) for fish of identical age. Reciprocals of reaction time multiplied by 100 ($100/(T-T_S)$) are indicated. Observed data from Fig. 25, lines fitted by eye.

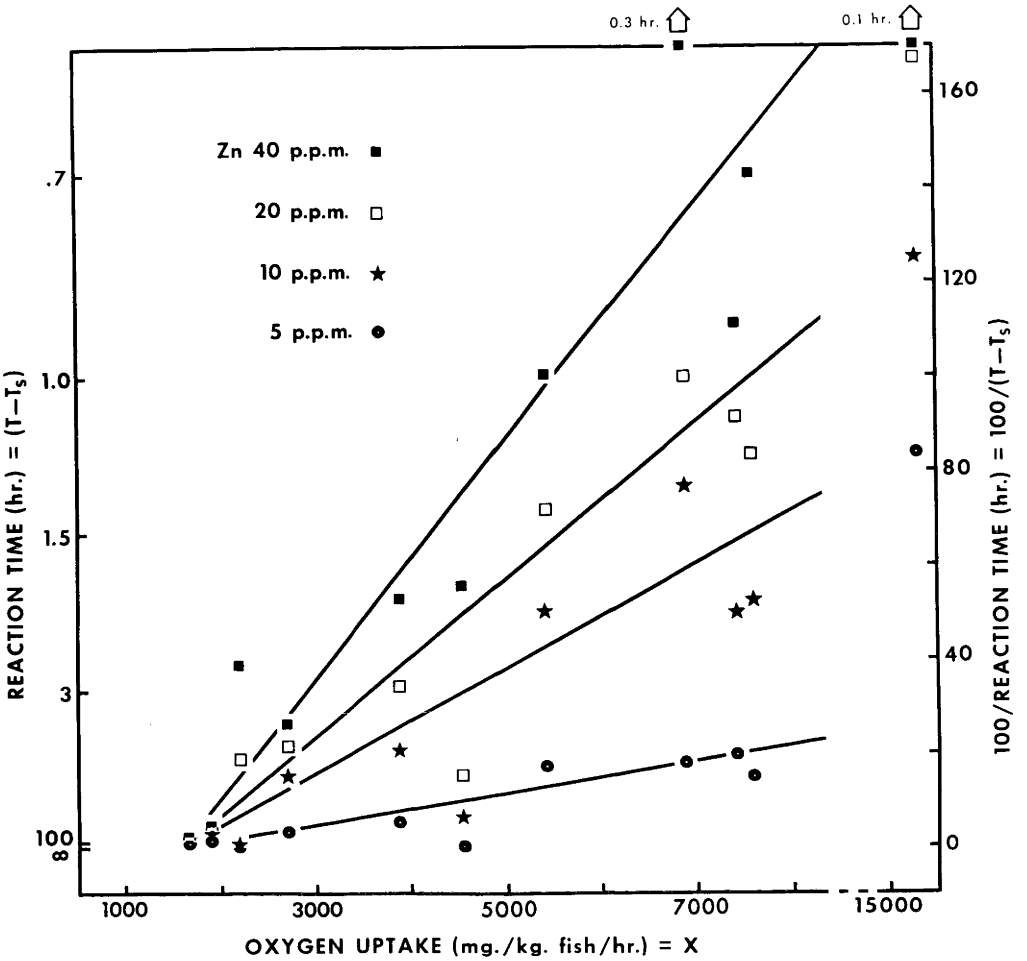

0.3 hr.
0.1 hr.
Zn 40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.
.7
1.0
1.5
3
100
80
160
120
80
40
0
1000
3000
5000
7000
15000
REACTION TIME (hr.) = (T−Ts)
100/REACTION TIME (hr.) = 100/(T−Ts)
OXYGEN UPTAKE (mg./kg. fish/hr.) = X

Table 10. Calculated values of the four constants in Equation 5, for four concentrations of zinc.

| Concentration of zinc (p.p.m. Zn) | $X_s$ (mg./kg. fish/hr.) | $T_s$ (hours) | n | K |
|---|---|---|---|---|
| 5 | 1600 | 1.3 | 1.0 | 30,000 |
| 10 | 1600 | 1.3 | 1.0 | 9,000 |
| 20 | 1600 | 1.3 | 1.0 | 6,000 |
| 40 | 1600 | 1.3 | 1.0 | 4,000 |

The curves drawn in Fig. 25 are fitted to these constants.

The discussion will now be extended further by considering the relationship between oxygen uptake and the size of the fish.

(iii) Resistance and body size

The absolute rate of oxygen uptake, measured in µl. per single zebrafish per hour, will be symbolized by Y, and the dry weight of the fish (measured in mg.) by W. If oxygen uptake (Y) is directly proportional to the weight of the fish (W), then

$$Y = WK_1 \qquad (6)$$

$$\text{and log. } Y = \text{log. } W + K_2 \qquad (7)$$

where $K_1$ and $K_2$ are constants. If oxygen consumption (Y) is proportional to the surface area of the fish $((\sqrt[3]{W})^2.K_3)$ then

$$Y = (\sqrt[3]{W})^2.K_4 \qquad (8)$$

$$\text{and log. } Y = \frac{2}{3} \text{ log. } W + K_5 \qquad (9)$$

where $K_3$, $K_4$ and $K_5$ are constants. If the data for a given species can be represented by Equation 7, the slope of the regression line of log. Y upon log. W will be 1. If the data can be represented by Equation 9, the slope will be $\frac{2}{3}$. An intermediate value for the slope would indicate that oxygen consumption is proportional to a function of size intermediate between surface and volume. Most studies reported by

Fry (1957) indicate a slope of about 0.8.

The data for zebrafish are presented in Fig. 21. The dry weights of fish aged from 5 to 13 days have been estimated both from Line C of Fig. 22 (heavy circles) and from Line B (light circles). The regression line has been fitted by eye to the heavy circles, excluding data for fish aged 0 to 4 days. The slope of the regression line is 0.85, and its equation

$$Y = 3.3\ W^{.85} \qquad (10)$$

Data for unhatched embryos fell below the line because embryos weighed more than newly hatched fish and used less oxygen. However, the slope of the regression line, and by inference the shape of the fish, remained fairly constant throughout most of the life cycle, across a range in size from 0.01 to 100 mg. dry weight. All previous studies appear to have been made using fish varying in size only about 100-fold from smallest to largest.

It has been demonstrated that reaction time in zinc ($T-T_S$) is inversely related to the relative rate of oxygen uptake per unit dry weight of fish (X), and that the absolute rate of oxygen uptake (Y) is directly related (from the fifth day) to a power of the dry weight of the fish (W). X and Y are related by the equation

$$X = \frac{1430\ Y}{W} \qquad (11)$$

It is apparent that T must be inversely related to a power of W, from the fifth day onwards. This relationship will now be considered.

By combining Equations 10 and 11, it can be shown that

$$X = 4720\ W^{-.15} \qquad (12)$$

By substituting values of $X_s$, $T_s$ and n from Table 10, and replacing X by $(4720\ W^{-.15})$, Equation 5 becomes

$$(4720\ W^{-.15} - 1600)(T - 1.3) = K \qquad (13)$$

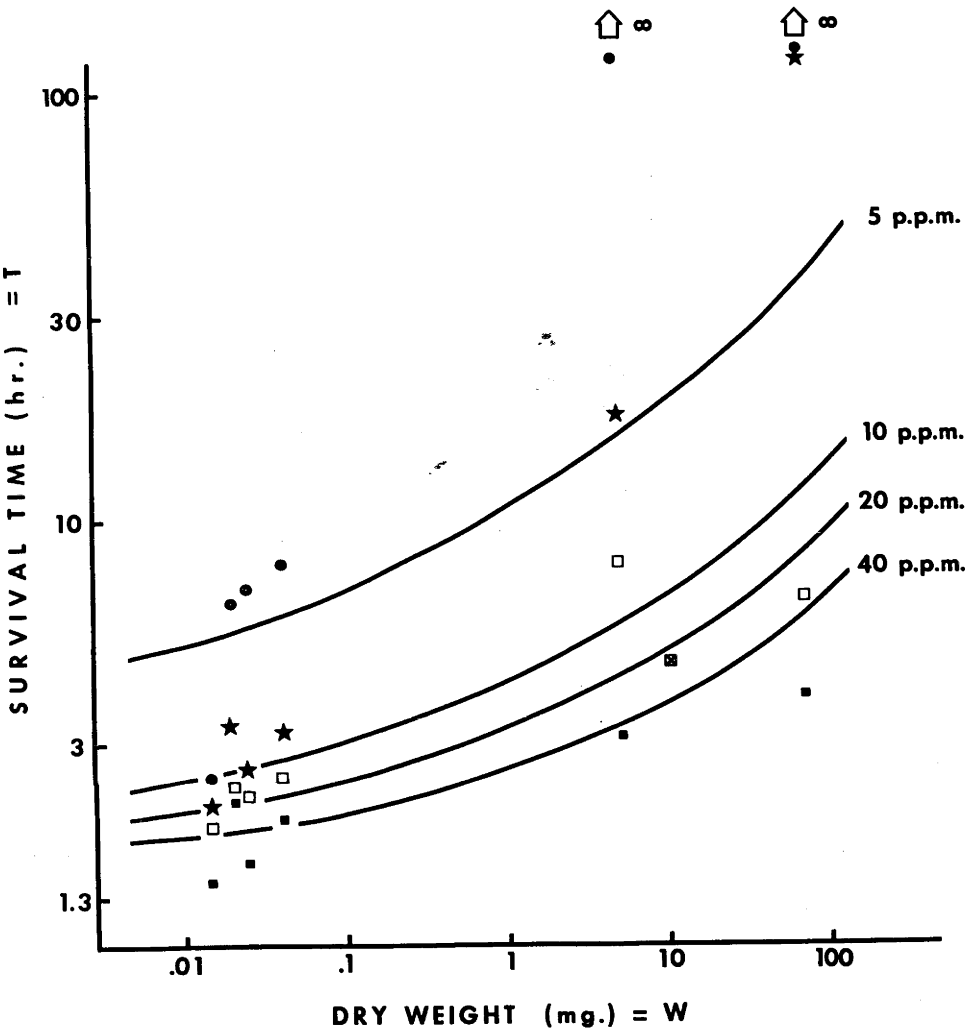
where K is a constant depending on the concentration of zinc (Table 10). Therefore

$$T = \frac{K}{(4720\ W^{-.15} - 1600)} + 1.3 \qquad (14)$$

The calculated regression lines of T upon W, for the four concentrations of zinc, are drawn in Fig. 28. Observed values of T and W are indicated.

It can be calculated from Equation 14 that when W = 0, T = 1.3 hours; and when T = $\infty$, W = 328 mg. Thus an infinitely small hatched zebrafish would be predicted to survive the threshold survival time ($T_s$) in all concentrations of zinc. On the other hand, a zebrafish having a dry weight of

Fig. 28. Survival time (T) on a reciprocal scale, of zebrafish in four concentrations of zinc, plotted against their dry weight (W). Regression lines calculated from Equation 14 and Table 10. Observed data indicated by ● (5 p.p.m. zinc), ★ (10 p.p.m.), □ and ⊠ (20 p.p.m.), and (40 p.p.m.). Reading from left to right, observed data plotted for fish aged 13, 10, 8, 6, 40, 78 and 100 days. Survival data for fish aged 78 days ( ⊠ ) from Table 13. Other survival data from Fig. 5. Weight data from Fig. 21 (heavy circles).


∞
∞
100
30
10
3
1.3
SURVIVAL TIME (hr.) = T
5 p.p.m.
10 p.p.m.
20 p.p.m.
40 p.p.m.
.01
.1
1
10
100
DRY WEIGHT (mg.) = W

328 mg. (which is beyond the normal range of size for the species) would be predicted from the equation to survive indefinitely in all concentrations of zinc.

In practice, of course, it would be foolhardy to make such predictions beyond the range of the observed data. For example, very high concentrations of zinc would be expected to kill large fish however much they weighed, by osmotic stress if by no other means.

The observed data for fish aged 6 to 13 days fit the theoretical curves in Fig. 28 fairly closely, at all four concentrations of zinc. The observed data for older fish, which were exposed to 20 or 40 p.p.m. zinc, fit the curves less closely. The data for older fish exposed to 5 or 10 p.p.m. zinc fall right away from the curves.

It will be shown in Section 7b (i) that the first toxic action of zinc on hatched zebrafish results in gill damage. Adult fish which survived about 12 hours in toxic solutions of zinc recovered from its toxic effects and survived indefinitely (Section 7c (i)). Thus any adult fish which may be predicted from Fig. 28 to survive more than 12 hours, may be expected from data in Section 7 to survive indefinitely. The major divergences of the observed data in Fig. 28 from the theoretical curves may thus be readily explained.

## 6. RELATION BETWEEN ZINC UPTAKE AND RESISTANCE

### a) Methods

#### (i) Rearing conditions

Zebrafish of common parentage were bred and reared in Canberra tap water, total hardness 10 p.p.m. $CaCO_3$, temperature 25°C., pH between 6.8 and 7.2, and dissolved oxygen concentration at least 6 p.p.m., that is, under conditions described in Section 3a. Fish were not starved before experiments.

#### (ii) Exposure to zinc

Six groups of zebrafish were exposed for different periods to 6-litre samples of tap water (total hardness initially $10 \pm 0.5$ p.p.m. $CaCO_3$) containing different concentrations of zinc. Most solutions were labelled with $10^{-2}$ μC. zinc-65 per ml. as chloride. Fish which survived at least 24 hours were fed sparingly on the second and subsequent days, for the duration of the experiment.

Three adult zebrafish (Group 1), total wet weight 832 mg., were exposed for 51 days to water containing initially

0.0068 p.p.m. Zn as chloride, labelled with zinc-65. After 50 days, the zinc concentration in solution or suspension had dropped to 0.0029 p.p.m. and the hardness had risen to 34 p.p.m. $CaCO_3$. Two more adult zebrafish (Group 2), combined wet weight 679 mg., were exposed for 51 days to water containing initially 0.007 p.p.m. Zn as sulphate, not labelled with zinc-65. After 51 days, the three fish exposed to zinc-65 were transferred to water containing 20 p.p.m. Zn as sulphate, which was not labelled with zinc-65. Concurrently, the two fish not previously exposed to zinc-65 were transferred to water containing 20 p.p.m. Zn as sulphate, which was labelled with zinc-65. All five fish were exposed to 20 p.p.m. zinc until immobilization.

Five adult zebrafish, and six juveniles aged approximately 50 days (Groups 3, 4 & 5), were exposed to water containing one of three concentrations of zinc sulphate, which were all labelled with zinc-65. Variable features of the exposures are listed in Table 11. After 48 hours, all three groups of test animals were transferred to plain tap water, which was changed daily for 9 days.

Nineteen juvenile zebrafish aged 78 days (Group 6), total wet weight 976 mg., were exposed until immobilization to water containing an initial concentration of 19.8 p.p.m. Zn as sulphate, labelled with zinc-65. The final

Table 11. Variable features of the procedure used to measure the zinc uptake of three groups of zebrafish.

| Group | Test fish | | Combined wet weight | Concentration of zinc in solution |
|---|---|---|---|---|
| | adults | juveniles | | |
| 3 | 2 | 2 | 656 mg. | 2.0 ± 0.2 p.p.m. |
| 4 | 2 | 2 | 677 mg. | 0.52 ± 0.01 p.p.m. |
| 5 | 1 | 2 | 211 mg. | 0.036 ± 0.002 p.p.m. |

concentration of zinc in solution or suspension was 19.2 p.p.m. Survival times of individual fish were noted.

(iii) Determination of zinc concentration

Zinc labelled with $Zn^{65}$ was detected using two 'Ekco' N664B scintillation counters, each connected to an 'Ekco' N610A automatic scaler. One counter was equipped with an 'Ekco' N597 sodium-iodide well crystal for well counting. The other was equipped with an 'Ekco' N566 cylindrical crystal, diameter 3.8 c.m., for 'end-on' counting. The activity of all samples (plus background) was measured by observing at least 10,000 counts, so that the estimated standard deviation never exceeded $\pm$ 1%.

Samples intended for radioassay were prepared in the following manner. Both live and dead fish were rinsed for 5 seconds in tap water. Live fish were transferred by net to beakers of diameter 3.8 cm. and height 3.0 cm. containing 15 ml. tap water, for 'end-on' counting. The water was free from zinc-65, but was chemically similar to the water from which the fish had been taken. Fish were returned to the experimental solutions after counting, and were weighed at the termination of experiments. Dead fish were blotted on filter paper and transferred to the bottom of weighed plastic vials which fitted into the well crystal. The vials were

then capped and reweighed. Water samples of approximately 1 ml. were transferred by Pasteur pipette to weighed vials, which were similarly capped and reweighed. Samples of water and fish were collected at appropriate intervals throughout the experiments.

The chemical concentration of zinc in solution, owing to the labelled zinc chloride, was estimated by polarographic assay of the stock solution. The initial zinc concentrations of experimental solutions containing approximately 0.5 or 2 p.p.m. Zn were similarly determined. Higher concentrations of zinc were analysed titrimetrically. Samples (1 ml.) of all experimental solutions containing zinc-65 were collected before the fish were added and set aside as standards. The uptake of zinc by fish, and changes of zinc concentration in solutions, were calculated by comparing the radioactivity of samples and standards.

b) Results

The uptake and loss of zinc labelled with zinc-65, by zebrafish in Groups 1 and 2, are summarized in Table 12. The uptake and loss by zebrafish in Groups 3, 4 and 5 are plotted in Fig. 29. The uptake by zebrafish in Group 6 is listed in Table 13. Concentrations of zinc in fish are calculated on a wet weight basis.

Table 12. Concentrations of zinc labelled with zinc-65, which were taken up by five adult zebrafish (Groups 1 & 2) during exposure to different concentrations of zinc in solution. All concentrations in parts per million.

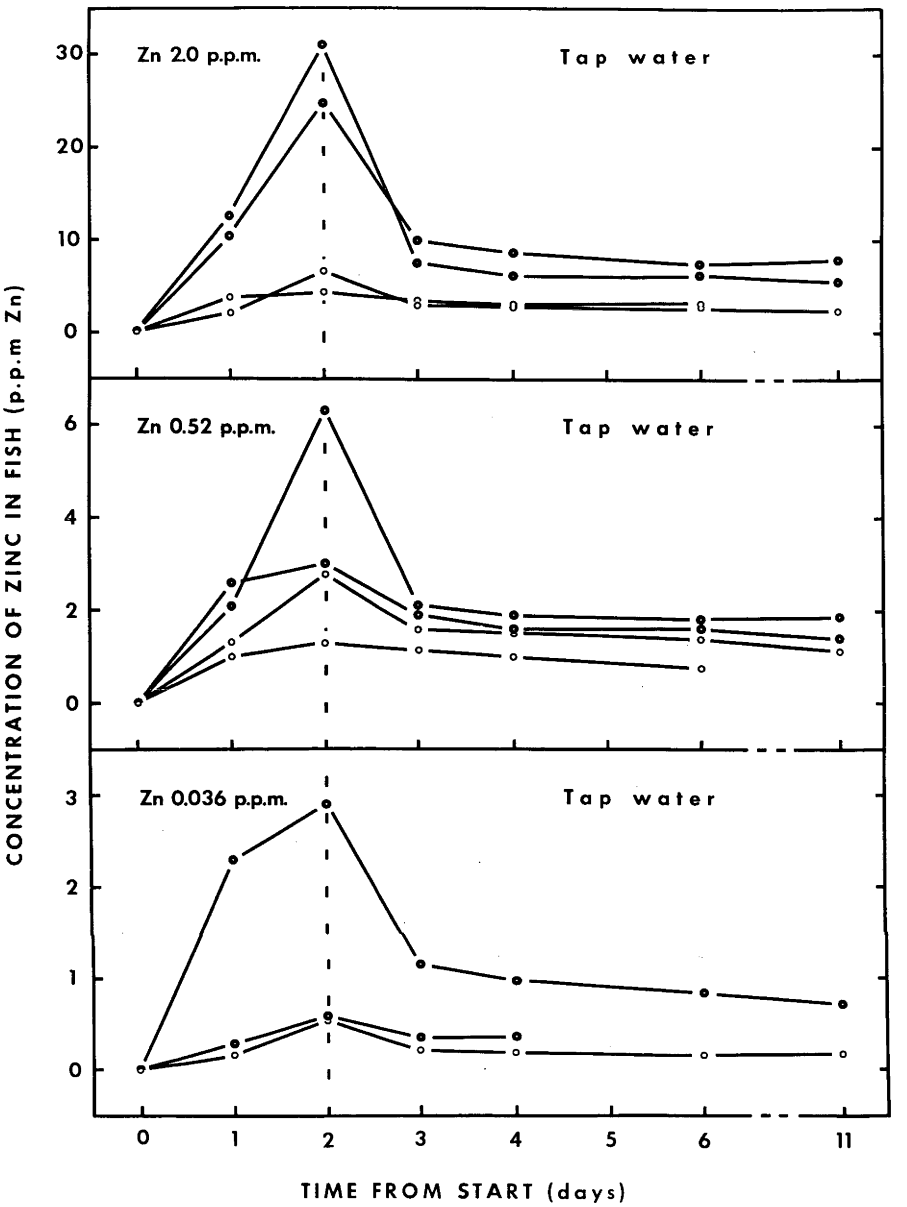
| Days from start | Group 1 | | | | Group 2 | | |
|---|---|---|---|---|---|---|---|
| | | Zinc labelled with zinc-65 in fish | | | | Zinc labelled with zinc-65 in fish | |
| | Zinc in solution | Fish 1 | Fish 2 | Fish 3 | Zinc in solution | Fish 1 | Fish 2 |
| 0 | 0.0068* | nil | nil | nil | 0.007 | nil | nil |
| 28 | 0.0029* | 1.3 | 0.85 | 2.1 | $<$0.007 | nil | nil |
| 50 | 0.0029* | 1.6 | 1.3 | 2.3 | $<$0.007 | nil | nil |
| 51 (at immobil.) | 20 | 1.3 | 0.65 | 2.2 | 20* | 64 | 55 |

* Zinc in solution labelled with zinc-65

Table 13. Survival time in tap water containing 19.5 ± 0.3 p.p.m. Zn, and uptake (on a wet-weight basis) of zinc labelled with zinc-65 at immobilization, by nineteen zebrafish aged 78 days (Group 6).

| Fish | Wet weight | Survival Time | Uptake of zinc labelled with zinc-65 at immobilization |
|---|---|---|---|
| 1 | 39 mg. | 3.3 hr. | 15.3 p.p.m. |
| 2 | 84 mg. | 3.6 hr. | 20.0 p.p.m. |
| 3 | 42 mg. | 3.9 hr. | 17.2 p.p.m. |
| 4 | 65 mg. | 4.2 hr. | 23.2 p.p.m. |
| 5 | 55 mg. | 4.2 hr. | 19.8 p.p.m. |
| 6 | 52 mg. | 4.3 hr. | 17.7 p.p.m. |
| 7 | 58 mg. | 4.4 hr. | 21.8 p.p.m. |
| 8 | 53 mg. | 4.4 hr. | 18.9 p.p.m. |
| 9 | 42 mg. | 4.5 hr. | 22.1 p.p.m. |
| 10 | 46 mg. | 4.6 hr. | 21.8 p.p.m. |
| 11 | 45 mg. | 4.8 hr. | 22.8 p.p.m. |
| 12 | 36 mg. | 4.8 hr. | 29.7 p.p.m. |
| 13 | 104 mg. | 4.8 hr. | 17.2 p.p.m. |
| 14 | 49 mg. | 5.0 hr. | 22.3 p.p.m. |
| 15 | 28 mg. | 5.0 hr. | 20.6 p.p.m. |
| 16 | 34 mg. | 5.0 hr. | 24.9 p.p.m. |
| 17 | 28 mg. | 5.0 hr. | 19.8 p.p.m. |
| 18 | 47 mg. | 5.1 hr. | 20.0 p.p.m. |
| 19 | 69 mg. | 5.1 hr. | 9.8 p.p.m. |

Fig. 29. Concentration on a wet-weight basis of zinc labelled with zinc-65, which was taken up by eleven zebrafish (Groups 3, 4, & 5) from tap water containing either 2.0, 0.52 or 0.036 p.p.m. zinc, and retained after transfer of the fish to zinc-free tap water. Heavy circles, fish aged 50 days. Light circles, adult fish.


Zn 2.0 p.p.m.
Tap water
Zn 0.52 p.p.m.
Tap water
Zn 0.036 p.p.m.
Tap water
CONCENTRATION OF ZINC IN FISH (p.p.m Zn)
TIME FROM START (days)
0
1
2
3
4
5
6
11

c) Discussion

The purpose of this discussion is to consider Hypothesis 3 of Section 3c, which is that the resistance of zebrafish to zinc poisoning is inversely proportional to the rate of whole-body zinc uptake. The rate of whole-body zinc uptake is here defined as the rate per unit time at which the concentration of zinc in (or on) the body increases on exposure of the fish to a solution of zinc. The concentration of zinc in the fish after exposure is equal to the concentration before exposure, plus the concentration taken up from the solution, minus the concentration of body zinc lost during exposure.

The loss of body zinc by three adult zebrafish has been shown to be negligible after their fatal exposure to a solution containing 20 p.p.m. zinc, whereas the uptake of zinc by two more zebrafish was substantial under similar conditions (Table 12). As the five fish were drawn from the same population, and were reared under similar conditions, it is concluded that the concentration of zinc in the fish, after immobilization in 20 p.p.m. zinc, was approximately equal to the concentration of body zinc before exposure plus the concentration of zinc taken up from the solution. It will be assumed later that the concentration of zinc taken up by

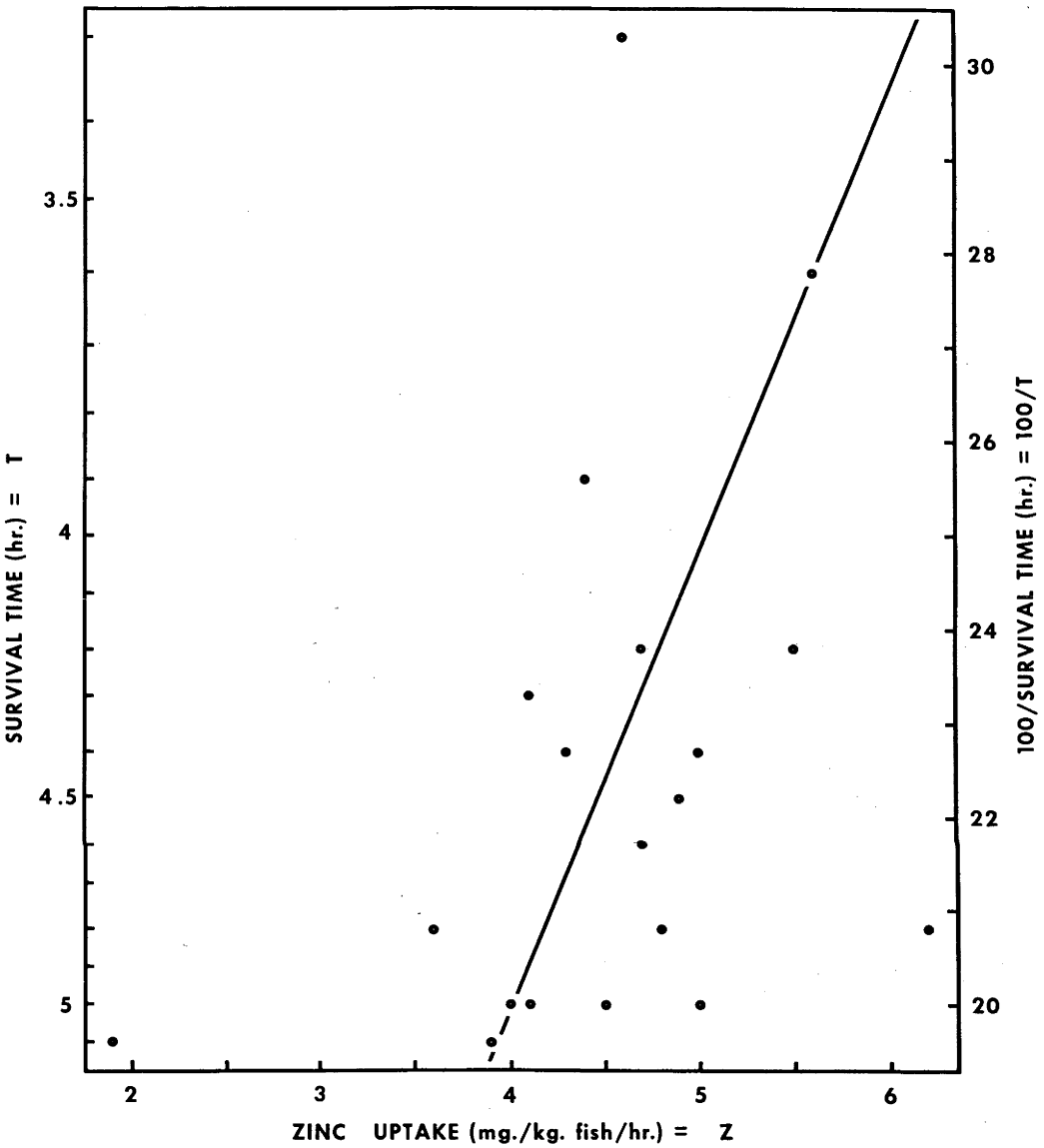
juvenile zebrafish, during fatal exposure to 20 p.p.m. zinc, can be calculated in a similar manner.

When six juvenile zebrafish and five adults (Fig. 29) were exposed to one of three non-lethal solutions of zinc (2.0, 0.54 or 0.036 p.p.m. Zn), the rate of uptake of zinc was fairly constant throughout 48 hours. It will be assumed in the following paragraph that the rate of uptake from a lethal solution of zinc remains constant until immobilization, during an exposure period of approximately 5 hours.

If the two assumptions mentioned above are made, Hypothesis 3 can be tested using the data presented in Table 13. The rate of whole-body zinc uptake is calculated by dividing the concentration of absorbed zinc by the survival time. The hypothesis is tested in Fig. 30, by plotting survival time on a reciprocal scale against the rate of zinc uptake. The reciprocal of survival time (multiplied by 100) is also shown. Data are presented for nineteen juvenile zebrafish, which were exposed until immobilization to $19.5 \pm 0.3$ p.p.m. zinc.

In the discussion which follows, the rate of zinc uptake, measured in mg. per kg. fish (wet weight) per hour, will be symbolized by Z, and survival time in hours (as in Section 5c (ii)) by T. If T is inversely proportional to Z (that is if $\frac{100}{T}$ is directly proportional to Z) the data in Fig. 30

Fig. 30. Survival time (T) on a reciprocal scale, of nineteen juvenile zebrafish in 19.5 ± 0.3 p.p.m. zinc, plotted against the rate of zinc uptake (Z). Reciprocals of survival time multiplied by 100 (100/T) are indicated. Data from Table 13. Line calculated from Equation 16.


SURVIVAL TIME (hr.) = T
3.5
4
4.5
5
2
3
4
5
6
ZINC UPTAKE (mg./kg. fish/hr.) = Z
100/SURVIVAL TIME (hr.) = 100/T
30
28
26
24
22
20

would fall along the indicated line. The equation of this line is

$$\frac{100}{T} = 5.0\ Z \qquad (15)$$

or

$$T = 20\ Z^{-1} \qquad (16)$$

the slope in Equation 15 being calculated from the mean values of the plotted data.

Owing to the wide scatter of the data, and especially to the positions of three outliers, there does not appear to be any statistical correlation between the two variables. Hypothesis 3 is therefore not supported by the available data. One explanation could be that the accuracy of the data is low. This possibility is however rejected because the maximum error in the survival data is calculated to be 6% and in the zinc-uptake data about 8%.

A more reasonable explanation is that the uptake of zinc is a highly complex process involving three possible sites of entry (gills, gut and skin). The heavy-metal metabolism of fish is poorly understood (Vallee, 1962), but it may be expected that zinc is transferred through the body by several interacting routes. Concurrently, some of the zinc may be excreted through the kidneys and (possibly) the gills. As the various processes involved in uptake, transfer and

excretion are likely to proceed at different rates, it is to be expected that the rates of whole-body zinc uptake of individual fish will vary widely. The situation is further complicated by the probability that gill permeability changes at time of immobilization.

In Section 7, it will be demonstrated that the toxic action of zinc is centred in the gills, at least when hatched fish are killed by a few hours' exposure to zinc. It is therefore possible that survival time may be primarily correlated with the rate of uptake of zinc by the gills, and only indirectly (if at all) with the rate of whole-body zinc uptake. An unsuccessful attempt was made to measure the uptake of zinc by the gills of zebrafish, but the attempt was abandoned because of the difficulty of collecting and weighing such minute pieces of tissue. If a larger species of test fish were used, the relationship between survival in lethal concentrations of zinc, and the rates of zinc uptake by the gills, would form a profitable area for further study.

## 7. TOXIC ACTION OF ZINC

### a) Methods

#### (i) Rearing conditions

Zebrafish of common parentage were bred and reared in Canberra tap water, total hardness 10 p.p.m. $CaCO_3$, temperature 25°C., pH between 6.8 and 7.2, and dissolved oxygen concentration at least 6 p.p.m., that is, under conditions described in Section 3a.

#### (ii) Exposure to zinc

Some of the observations which will be reported in Section 7b were made on selected test fish used in the toxicit bioassays described in Section 3. In addition, samples of about 12 juvenile fish aged 25-37 days were exposed to 6-litre volumes of water containing 5, 3, 2.5, 2, 1.3, 1, or 0 p.p.m. zinc, respectively. Solutions were aerated by air diffusers. About 90% of the solutions were replaced on the third day and thereafter weekly. Fish were fed daily.

(iii) Preparation of specimens

Test fish which were in the process of being killed rapidly by zinc poisoning were usually collected at or near the time of immobilization. Fish which survived more than one day were collected after appropriate periods of exposure. In addition, any test fish was collected and preserved if it became deformed, or if it was observed to swim with difficulty or to lose its balance. Dead fish were not collected.

Some fresh material, especially unhatched embryos, was examined microscopically. Fish intended for histological examination were fixed in Bouin's solution. The tail was severed from the body of adult fish and juveniles, in the vicinity of the anus, to speed fixation. Preserved fish were stored in Bouin's solution. The bones of adult fish were decalcified by formic acid diluted in Ringer's solution (Baker, Silverton & Luckcock, 1962). Fish were dehydrated, cleared in methyl benzoate, mounted in paraffin wax (melting point 12-14°C.), and sectioned at 5-8 $\mu$. Sections were cut transversely from the head end or longitudinally from the left side. They were either stained with haematoxylin and eosin, or treated by the periodic acid - Schiff reaction (Pearse, 1960, p. 832) and stained with haematoxylin. Sections were permanently mounted in 'DePeX' and studied

microsocopically. Gills, liver, kidneys, pancreas, spleen, gonads, gut, brain, skin, pituitary and muscle were routinely examined.

b) Results

Histological observations on thirty-eight experimental fish and twenty-three controls are listed in Tables 15 and 14, respectively.

(i) Gill damage

Seven fish aged 6, 13, 38 and approximately 100 days were immobilized by zinc in 7 hours or less. All suffered gill damage. All fish surviving 29 hours or longer were free from gill damage as described below. No fish dying between 7 and 29 hours were examined histologically.

In fish of all ages, gill damage followed the same pattern (Figs. 31 to 33). At immobilization, the epithelium covering the gill lamellae was sloughed off from approximately three-quarters of the total gill surface. It formed a detached mass of tissue, separated from the underlying tissues of the gill, which were apparently undamaged. Blood vessels showed no sign of haemorrhage. Photomicrographs of gills from control fish are shown in Figs. 34 and 35.

Sections of zebrafish treated by the PAS reaction demonstrated the distribution of polysaccharides (Figs. 31 to 35).

Table 14. List of zebrafish reared in tap water and examined histologically.

| Reference number | Age when preserved | Remarks* |
|---|---|---|
| | days | |
| 1 | 0.4 | Blastoderm over 3/4 yolk |
| 2 | 1.5 | Lens of eye developed |
| 3 | 2.5 | Slits to side of pharynx. No gills |
| 4 | 3.4 | 4 gills, gill slits open. Mucus glands in skin, not in pharynx |
| 5 | 4.2 | Gut open, mouth to anus |
| 6 | 5.1 | Pituitary. Pronephros. Pancreas |
| 7 | 6.1 | Numerous mucus glands in pharynx |
| 8 | 6.2 | Control for Fish 24-28 |
| 9 | 6.2 | Control for Fish 24-28 |
| 10 | 13 | Control for Fish 29-31 |
| 11 | 13 | Control for Fish 29-31 |
| 12 | 39 | Control for Fish 32-34 |
| 13 | 43 | Control for Fish 35-40 |
| 14 | 43 | Control for Fish 42 |
| 15 | 46 | Control for Fish 41 |
| 16 | 48 | Control for Fish 43-45, 48, 49, 51 |
| 17 | 53 | Control for Fish 46 |
| 18 | 53 | Control for Fish 47, 52 |
| 19 | 62 | Undersized. Control for Fish 50, 53 |
| 20 | 86 | Undersized. Control for Fish 54 |
| 21 | 149 | Control for Fish 55 |
| 22 | 100+ | Gravid ♀. Control for Fish 56-61 |
| 23 | 100+ | Mature ♂. Control for Fish 56-61 |

* Fish normal and typical of sample from which drawn, unless otherwise noted.

Table 15. List of zebrafish which were examined histologically after being exposed to tap water containing zinc sulphate.

| Reference number | Age when preserved | Concentration of zinc as Zn | Exposure time | Condition when preserved | Changes in tissues* |
|---|---|---|---|---|---|
| | days | | | | |
| 24 | 6.2 | 20 p.p.m. | 1.0 hr. | active | - |
| 25 | 6.2 | 20 p.p.m. | 1.5 hr. | active | Gill damage slight |
| 26 | 6.2 | 20 p.p.m. | 1.5 hr. | active | - |
| 27 | 6.2 | 20 p.p.m. | 2.0 hr. | nearly immobilized | Gill damage severe |
| 28 | 6.2 | 5 p.p.m. | 6.1 days | immobilized | - |
| 29 | 13 | 40 p.p.m. | 1.4 hr. | immobilized | Gill damage severe |
| 30 | 13 | 20 p.p.m. | 1.5 hr. | immobilized | Gill damage severe |
| 31 | 13 | 2.5 p.p.m. | 13 days | active | - |
| 32 | 38 | 20 p.p.m. | 7.1 hr. | immobilized | Gills damaged, not clogged with mucus. Mucus glands in pharynx discharged |
| 33 | 38 | 20 p.p.m. | 7.1 hr. | immobilized | Gill damage severe |
| 34 | 39 | 1 p.p.m. | 14 days | active | - |
| 35 | 41±5 | 20 p.p.m. | 29 hr. | immobilized | Gills swollen |
| 36 | 43 | 2.5 p.p.m. | 10 days | overturned | Gills swollen |

Table 15 (cont.)

| | | | | | |
|---|---|---|---|---|---|
| 37 | 43 | 2.5 p.p.m. | 10 days | overturned | Liver damage slight |
| 38 | 46 | 5 p.p.m. | 13 days | overturned, large fish | - |
| 39 | 46 | 5 p.p.m. | 13 days | overturned, undersized | - |
| 40 | 46 | 5 p.p.m. | 13 days | overturned, undersized | Liver damage slight. Gills swollen |
| 41 | 46 | 1 p.p.m. | 21 days | active | - |
| 42 | 47 | 5 p.p.m. | 14 days | overturned | Liver damage slight. Gills swollen |
| 43 | 48 | 3 p.p.m. | 11 days | moribund | - |
| 44 | 48±5 | 10 p.p.m. | 8 days | moribund | Liver damage intermediate. Gills swollen. Kidney tubules expanded |
| 45 | 49 | 2 p.p.m. | 12 days | moribund | Liver damage slight |
| 46 | 53 | 1 p.p.m. | 28 days | active | Liver damage slight |
| 47 | 55 | 2.5 p.p.m. | 22 days | overturned | Liver damage slight. Gills swollen |
| 48 | 57±5 | 5 p.p.m. | 17 days | active | - |
| 49 | 59 | 3 p.p.m. | 21 days | active, growth on belly | Liver damage slight. Cysts in muscle, probably protozoan |

Table 15 (cont.)

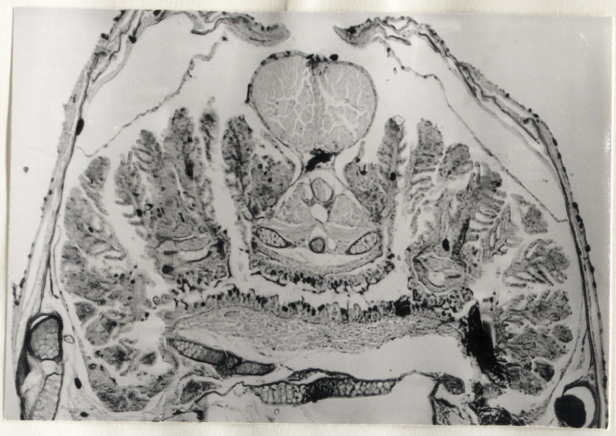
| | | | | | |
|---|---|---|---|---|---|
| 50 | 59 | 2.5 p.p.m. | 26 days | overturned | Liver damage severe. Gills swollen. Epithelium of pharynx sloughed off |
| 51 | 59 | 1 p.p.m. | 21 days | active, undersized | Liver damage slight |
| 52 | 62 | 1.3 p.p.m. | 29 days | losing balance | Liver damage slight. Gills swollen |
| 53 | 63 | 2.5 p.p.m. | 26 days | overturned | Liver damage severe. Gills swollen |
| 54 | 86 | 1.3 p.p.m. | 53 days | overturned | Liver damage severe |
| 55 | 149 | 1.3 p.p.m. | 116 days | stunted, tail deformed | Liver damage slight. Spine histologically normal |
| 56 | 100+ | 40 p.p.m. | 2.2 hr. | immobilized | Gill damage severe |
| 57 | 100+ | 10 p.p.m. | 45 days | active | - |
| 58 | 100+ | 10 p.p.m. | 45 days | active | - |
| 59 | 100+ | 10 p.p.m. | 82 days | active | - |
| 60 | 100+ | 10 p.p.m. | 82 days | swimming feebly | - |
| 61 | 100+ | 5 p.p.m. | 6 hr. | immobilized | Gill damage severe |

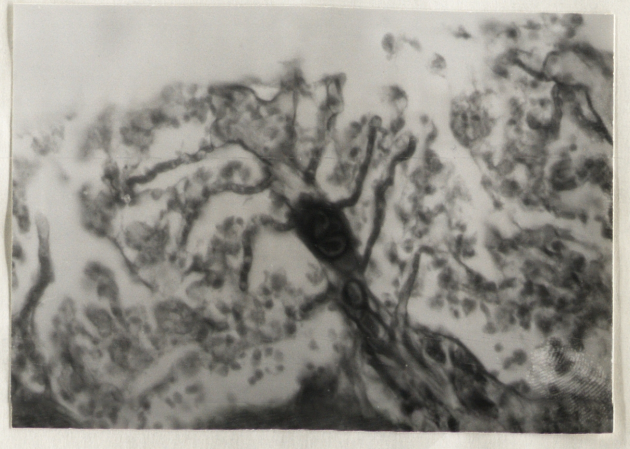
* Tissues similar to those of control fish unless otherwise noted. See Table 14

Fig. 31. T.S. through pharyngeal region of juvenile zebrafish which had been immobilized after 7.1 hours' exposure to 20 p.p.m. zinc (Table 15, Fish 32). Most gill lamellae damaged. Most mucus glands undischarged. Slight mucus discharge in pharynx. Stain: PAS + H. Magnification x58.

Fig. 32. Damaged gill lamellae from same fish (Table 15, Fish 32). Note intact vascular endothelium and detached epithelial tissue. No mucus present. Stain: PAS + H. (x370)

Fig. 33. Damaged gill lamellae from same fish (Table 15, Fish 32). Strand of mucus (away from detached tissues and gill remnants) indicated by arrow. Stain: PAS + H. (x370)





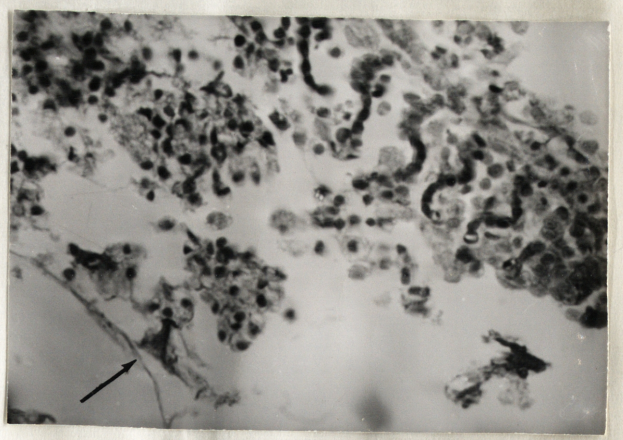
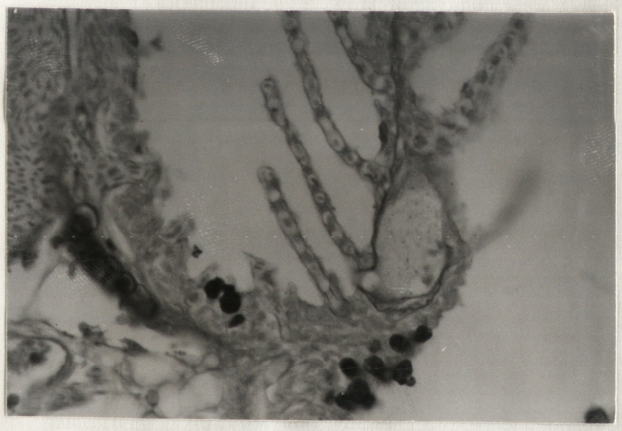


Fig. 34. T.S. through pharyngeal region of juvenile zebrafish not exposed to zinc (Table 14, Fish 12). Stain: PAS + H. (x23)

Fig. 35. Gill lamellae from same fish (Table 14, Fish 12). Note mucus glands. Stain: PAS + H. (x370)



In particular, the treatment revealed the location of mucus glands in the buccal cavity, pharynx, gills, opercular chamber gut and skin. Most other tissues were also coloured by the PAS reaction, owing especially to the presence of glycogen.

Discharged mucus glands were observed in the gut walls of all fish, including controls. Apart from those in the gut discharged glands were only observed in one of the seven fish suffering gill damage, and Figs. 31 to 33 illustrate sections from this particular fish. Glands in the walls of the pharynx were affected. Traces of discharged mucus were detected both in the pharynx and in the opercular cavity (Figs. 31 & 33). Mucus was not mixed with the sloughed-off epithelial tissue, nor did it cover the gill remnants (Figs. 31 & 32). It is concluded that gill damage was not aggravated by the clogging of gills with mucus.

(ii) Liver damage

Damage to the liver was observed in nine out of fourteen juvenile fish, which were immobilized or overturned or had become moribund, after exposure to zinc for 8 days or longer (Table 15). Four out of seven active juveniles also showed liver damage, after exposure to zinc for 8 days or longer. No liver damage was observed in fish which were adult before exposure, nor in fish aged 13 days or less.

Liver damage was characterized by the disintegration of polyhedral cells and by the development of intercellular spaces (Figs. 36 to 38). Destroyed cells tended to be randomly distributed (Fig. 36). Intercellular spaces developed either between individual cells (Fig. 37) or between clumps of cells (Fig. 38). Photomicrographs of the liver in a control fish are shown in Figs. 39 and 40.

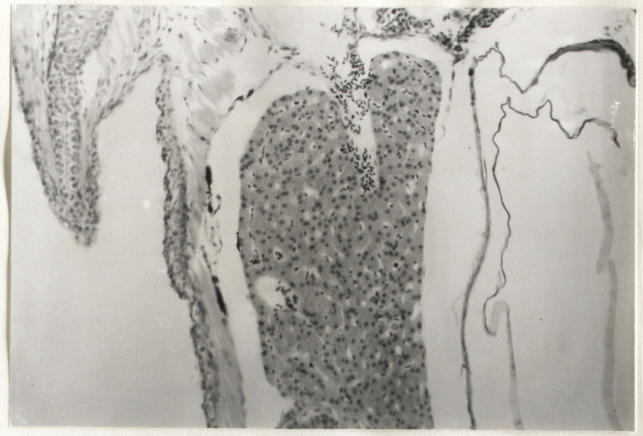
In most juvenile zebrafish immobilized after prolonged exposure to zinc, liver damage was associated with oedema of the gill lamellae (Figs. 41 & 42). The two effects may have been part of the same syndrome. The oedema resulted from an increase in volume of the intercellular space between the respiratory epithelium of the lamellae and the vascular endothelium, the effect being to lift the epithelium away from the underlying tissues. In every affected fish, at least three-quarters of the observed gill lamellae appeared swollen. Identical swellings were observed in control fish, but never more than 1% of the total lamellae were affected.

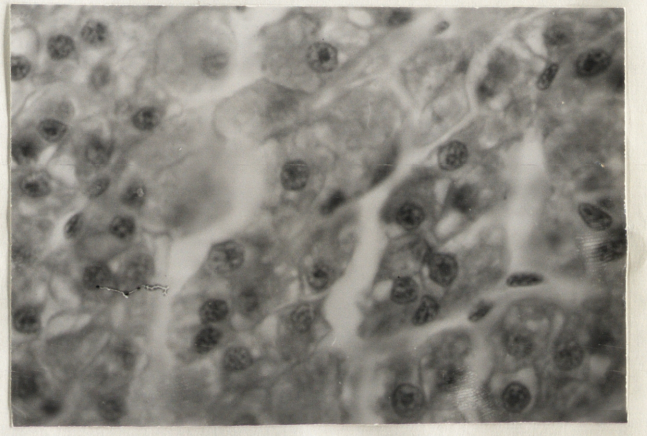
No other histological changes were generally associated with liver damage, but kidney tubules were enlarged in one fish, and the epithelium of the pharynx had been sloughed off in another. Cysts in the muscle of a third fish were attributed to a protozoan infection.

Fig. 36. Liver (centre of field) from juvenile zebrafish which had overturned after 26 days in 2.5 p.p.m. zinc (Table 15, Fish 53). Note intercellular spaces, apparently resulting from destruction and removal of polyhedral cells. Stain: HE. (x150)

Fig. 37. Liver, detail of Fig. 38. Note spaces between individual cells. (x920)

Fig. 38. Liver (centre of field) from juvenile zebrafish which had been exposed to 1 p.p.m. zinc for 28 days (Table 15, Fish 46). Note spaces between clumps of cells. Stain: HE. (x150)





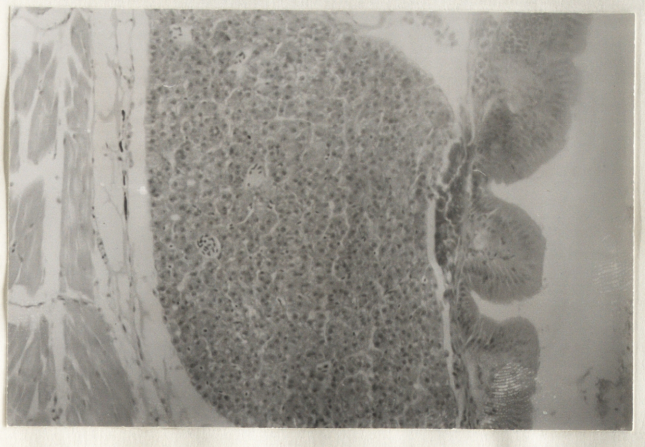
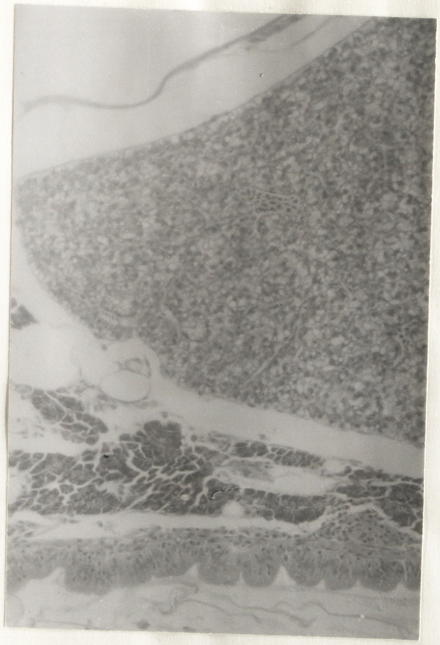


Fig. 39. Liver (centre of field) from juvenile zebrafish not exposed to zinc (Table 14, Fish 17). Stain: HE. (x150)

Fig. 40. Liver from another control zebrafish (Table 14, Fish 18). Stain: HE. (x920)



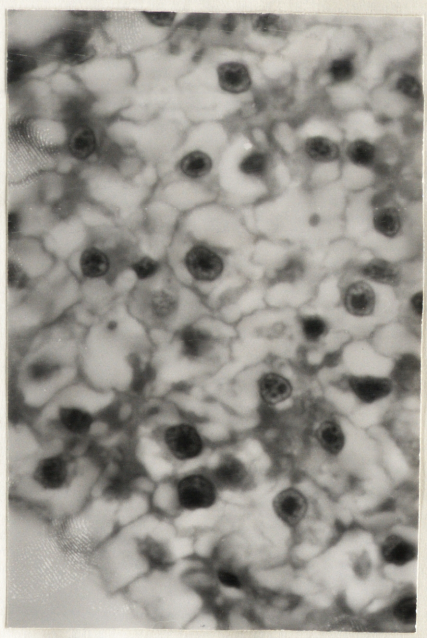
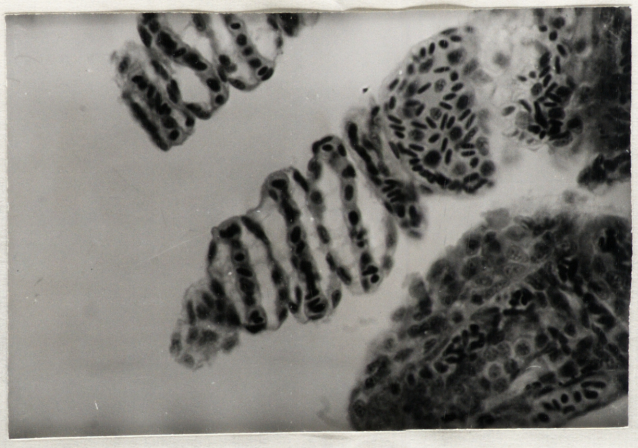
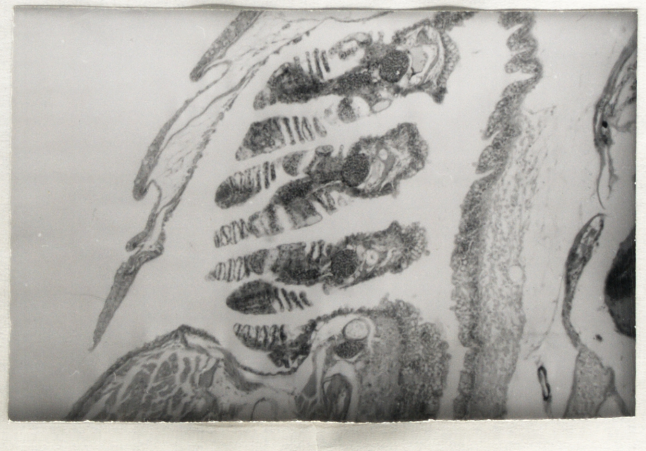


Fig. 41. L.S. through pharyngeal region of juvenile zebrafish which had overturned after 26 days in 2.5 p.p.m. zinc (Table 15, Fish 53). Note oedema of most gill lamellae. Stain: HE. (x58)

Fig. 42. Gill lamellae, detail of Fig. 41. Note respiratory epithelium lifted away from vascular endothelium. (x370)





(iii) Other damage

The toxic action of zinc on unhatched embryos, prior to their immobilization, has been described in Section 4c (ii) and illustrated in Figs. 13 to 15. When embryos were exposed to concentrations of zinc ranging from 5 to 1000 p.p.m. development continued normally until the embryos were at leas 36 hours old. They then became immobilized in a few hours. Patches of opaque material formed first in the perivitelline fluid of the egg, and later in the tissues of the embryo itself. Patches quickly coalesced until the fish became immobilized.

Unhatched embryos exposed to 3000 p.p.m. zinc for a few hours shrank in size and died rapidly.

c) Discussion

The purpose of this section is to consider Hypothesis 4 of Section 3c, which is that variations in the resistance of zebrafish to zinc poisoning are associated with differences in the toxic action of zinc.

(i) Toxic action of zinc

Four different modes of toxic action of zinc can be recognized by their histological effects. These are, firstly

breakdown of the gill epithelium; secondly, liver damage and associated oedema of the gills; thirdly, formation of opaque patches in the tissues; and fourthly, tissue shrinkage.

Unhatched embryos were vulnerable to the third action, which was slow, and (at extremely high concentrations of zinc to the rapid fourth action. They naturally escaped gill damage until they developed gills on the third day.

All fish possessing gills were vulnerable to gill damage if the concentration of zinc was high enough to kill the fish within a few hours. If they survived this critical period, which probably varied according to the size of the fish (Section 5c (iii)), they avoided the first toxic action altogether.

Juvenile fish surviving the first toxic action of zinc then became liable to the second. Newly hatched fish may have been similarly affected, but no histological evidence was observed. Fish normally survived in 2.5 or 1.3 p.p.m. zinc until they were 13 days old, at which time both experimental and control animals died of starvation. It is probable that the exposure time was too short for the second toxic action of zinc to cause histological changes.

Finally, it should be noted that adult fish which survived the first toxic action of zinc (culminating in gill damage) suffered no further mortality whatever. Fish continued to swim vigorously and eat normally in 5 or 10 p.p.m.

zinc for up to 84 days. Preserved fish showed no histologica abnormalities. Upon transfer of experimental fish back to tap water, eighteen of them came into breeding condition within a few days, and at least eight spawned successfully to produce normal offspring. It is therefore concluded that the test concentrations of zinc were non-toxic to adult zebrafish which were able to survive the initial toxic effect.

The conditions under which the different kinds of toxic action were observed are summarized in Table 16. It is apparent that juvenile zebrafish were vulnerable to the toxic action of zinc in two ways, depending on concentration. All other age groups of fish were only vulnerable to the toxic action of zinc in one way, except at very high concentrations

Variations in the resistance of the zebrafish to zinc poisoning, at different stages of its life history, have been summarized in Fig. 5. Comparison of Fig. 5 and Table 16 indicates that variations in resistance may be associated wit different patterns of dying. The low resistance of all hatched fish to 20 or 40 p.p.m. zinc was associated with gill damage at immobilization. The high resistance of unhatched embryos to all four concentrations of zinc was associated wit a toxic action producing patches of opaque material within the body (and avoidance of gill damage). The intermediate resistance of some juvenile fish to 5 p.p.m. zinc was related

Table 16. Toxic action of zinc on zebrafish of different ages, classified by the resulting histological changes.

| Age of fish | Concentration of zinc | Survival time | Toxic action |
|---|---|---|---|
| 0-3 days | 3000 p.p.m. | $<$10 hr. | decrease in volume of embryo |
| 0-3 days | 5-1000 p.p.m. | variable | patches of tissue turned opaque |
| 6-100 days | 5-40 p.p.m. | $<$12 hr. | gill damage |
| approx. 40 days | 1.3-5 p.p.m. | $>$7 days | liver damage and swollen gills |

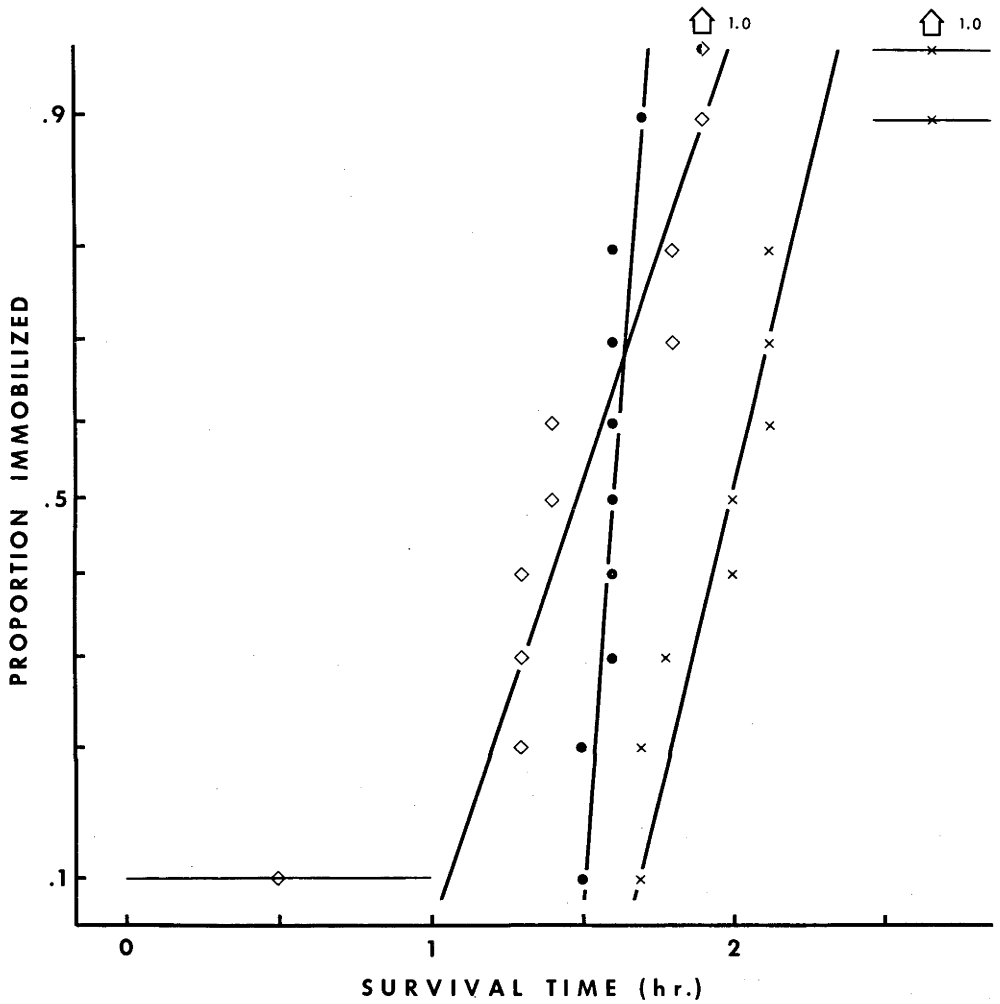
to liver damage. The indefinite survival of some adult fish in 10 p.p.m. and of most adults in 5 p.p.m. was associated with a complete absence of any histological change. The available data therefore support Hypothesis 4.

(ii) Probit analysis of toxicity data

In Section 3, the median survival times of samples of approximately ten zebrafish, of the same age and subjected to the same treatment, were usually estimated graphically by probit analysis. Regression lines were in most cases linear when a suitable transformation of time - such as logarithms - was selected. From this may be inferred that survival time (or the appropriate transformation) was normally distributed. This would be expected to occur when a sample of fish succumbed to a single toxic action of zinc.

An example of such a linear regression line is given in Fig. 43. The graph shows the cumulative mortality on a probit scale of three series of 8-day-old zebrafish (thirty fish altogether) in 40 p.p.m. zinc, plotted against simple survival time. The median value of these data (1.7 hours) is plotted in Fig. 5. Most of the toxicological data reported in Section 3 could be fitted to linear regression lines similar in form to those in Fig. 43. The data for juvenile fish in 5 p.p.m. zinc, and for adults in both 5 and 10 p.p.m. zinc, could not be so fitted.

Fig. 43. Cumulative mortality on a probit scale, of three series of zebrafish exposed to 40 p.p.m. zinc in tap water, plotted against survival time. Age of fish, 8 days. Median survival time (1.7 hours) plotted in Fig. 5. Horizontal lines indicate periods during which fish became immobilized. Periods $< 0.2$ hr. not indicated.


1.0
1.0
.9
.5
.1
PROPORTION IMMOBILIZED
0
1
2
SURVIVAL TIME (hr.)

A discontinuity in the plot of cumulative mortality (on a probit scale) against survival time (on any suitable scale) would indicate that the distribution of survival time was bimodal. In this case, the individual terms could be considered to have been drawn from two separate populations of survival time. A 'split probit' of this type would be expected when part of a sample of fish was killed by one kind of toxic action and the survivors by a second toxic action proceeding at a different - and necessarily slower - pace.

When the cumulative mortality of juvenile zebrafish in 5 p.p.m. zinc is plotted against log. survival time, such a split probit results (Fig. 44). Data for the three series of experiments reported in Section 3 (twenty-nine fish in all) have been pooled to produce the graph.

The regression line of cumulative mortality upon log. survival time, for adult zebrafish exposed to 10 p.p.m. zinc, is shown in Fig. 45. Data for the three series of experiments reported in Section 3 (twenty-nine fish) have been pooled. In all three series, fish surviving the first few hours' exposure to zinc were able to survive indefinitely. The mortality of fish exposed to 5 p.p.m. zinc followed a similar pattern, except that fewer died.

There are thus some interesting parallels between the different kinds of recognisable toxic action and the mortality

Fig. 44. Cumulative immobilization on a probit scale, of three series of zebrafish exposed to 5 p.p.m. zinc in tap water, plotted against survival time on a logarithmic scale. Data for the three series pooled. Age of fish 40 ± 5 days. Horizontal lines indicate periods during which fish became immobilized.

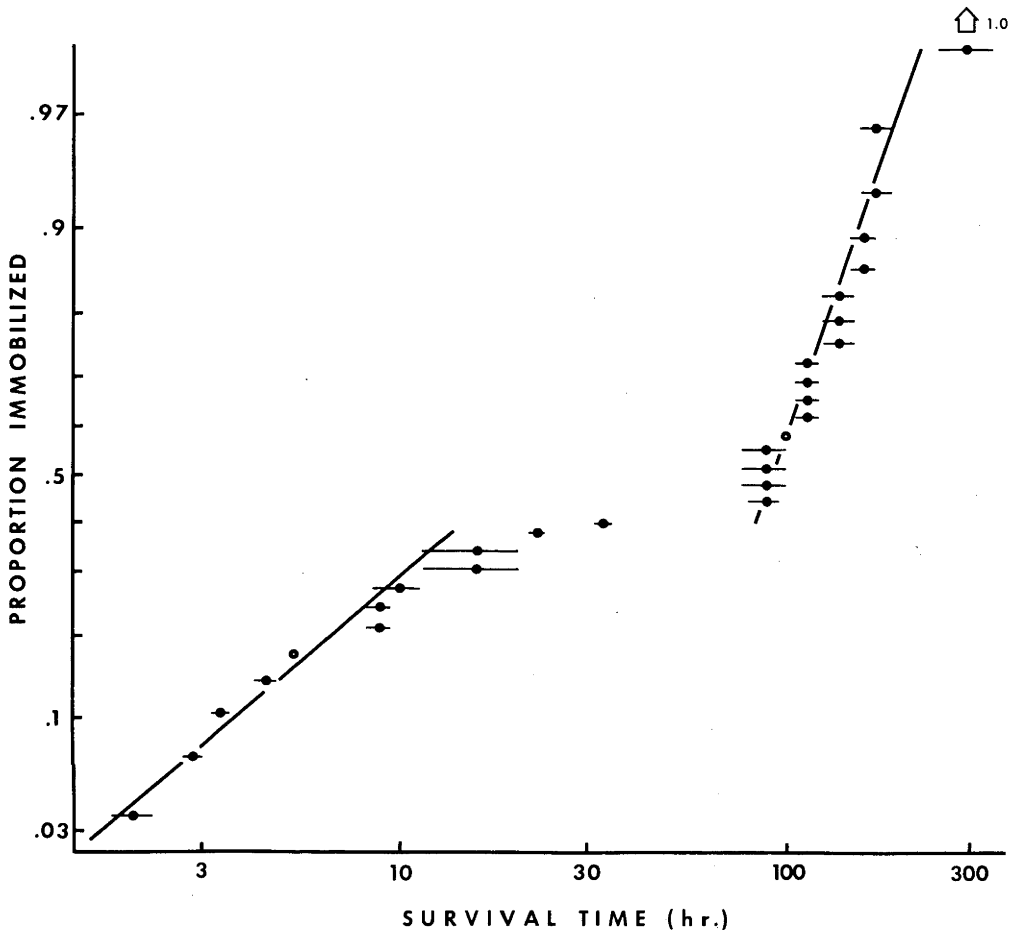
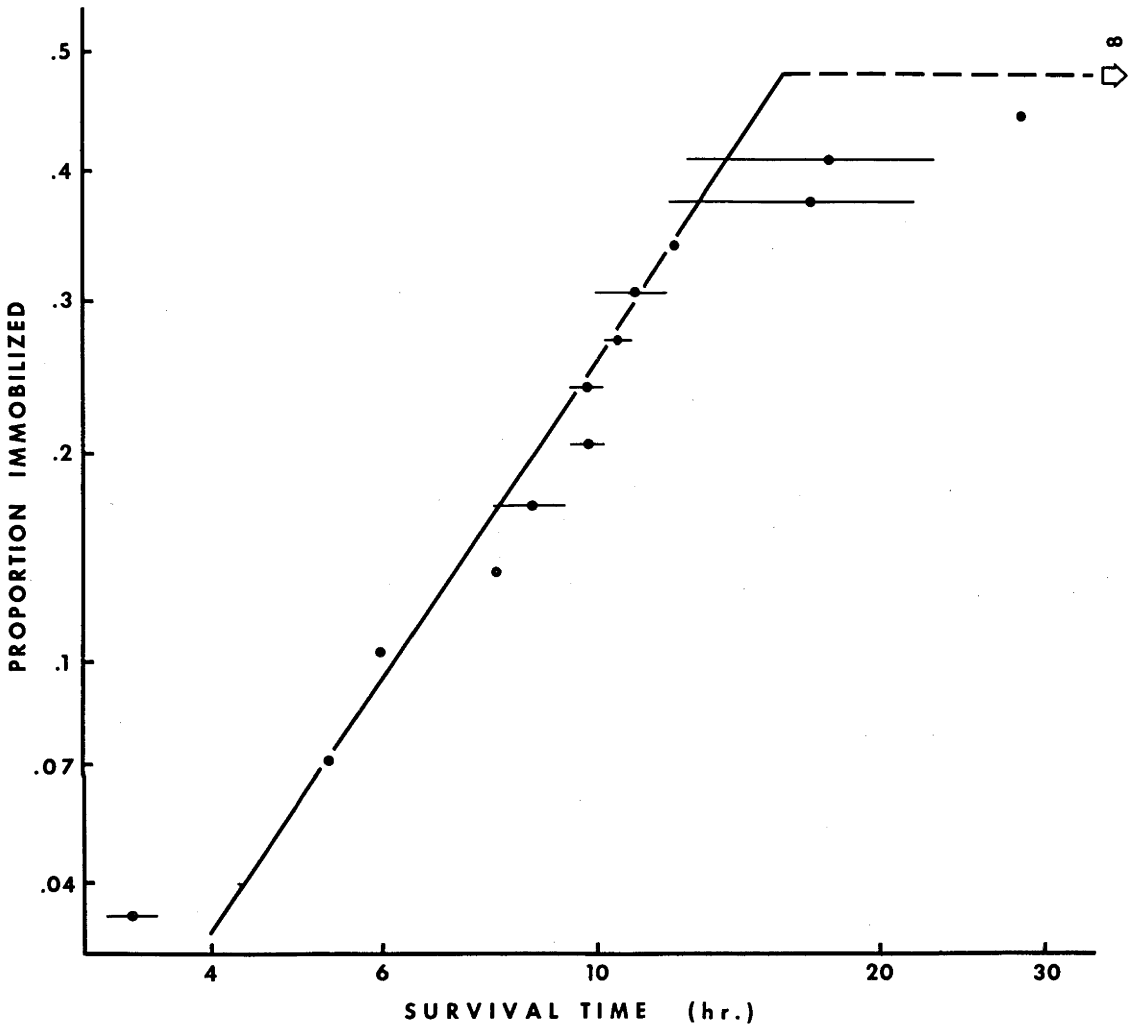

1.0
.97
.9
.5
.1
.03
PROPORTION IMMOBILIZED
3
10
30
100
300
SURVIVAL TIME (hr.)

Fig. 45. Cumulative immobilization on a probit scale, of three series of zebrafish exposed to 10 p.p.m. zinc in tap water, plotted against survival time on a logarithmic scale. Data for the three series pooled. Age of fish approximately 100 days. Horizontal lines indicate periods during which fish immobilized.


.5
.4
.3
.2
.1
.07
.04
PROPORTION IMMOBILIZED
4
6
10
20
30
SURVIVAL TIME (hr.)
∞

patterns in toxicological experiments. In most experiments, the survival time of test fish was normally distributed, and only one kind of toxic action was detected. The survival data of adults showed partial mortality in 5 or 10 p.p.m. zinc and histological examination revealed the survivors to be completely undamaged by zinc. Juveniles formed the only age group illustrating split probits. They also showed the results of two entirely different kinds of toxic action. The observations summarized in this paragraph reinforce the conclusions in the final paragraph of Section 7c (i). Hypothesis 4 of Section 3c (that variations in resistance are associated with differences in toxic action) is thereby further supported.

# 8. GENERAL DISCUSSION

The survival time of zebrafish in a given concentration of zinc sulphate has been taken as a measure of resistance. Survival time has been shown to vary with concentration (Section 3), with age (Section 3), and with the rate of oxygen uptake per unit dry weight of fish (Section 5). Because oxygen uptake is a function of the size of the animal, survival time has also been expressed as a function of dry weight (Section 5). More briefly,

$$T = f ( A, X, W, C ) \qquad (17)$$

where T, A, X, W and C are survival time, age of fish, oxygen uptake, dry weight, and concentration of zinc, respectively. It was anticipated that survival time would vary with the rate of whole-body zinc uptake, but this was not found to be the case (Section 6). It was expected that the high resistance of unhatched embryos might be owing to protection by the chorion, but the chorion proved to be a liability to the embryo rather than an asset (Section 4). On the other hand, variations in resistance were associated with differences in the mode of toxic action of zinc, which were parallelled by

differences in the pattern of mortality revealed by probit analysis (Section 7).

The various factors discussed in Sections 3 to 7 will now be considered in turn, in relation to the main body of literature summarized in Section 2.

The relationship between survival time and age of fish (T & A) is complex (Figs. 4 & 5), and the prime purpose of this thesis is to interpret the results of Section 3 in terms of other variables. The literature contains no relevant life history studies with which the results of Section 3 may be usefully compared.

The relationship between survival time and oxygen uptake (T & X) is

$$(T - T_S)\ (X - X_S)^n = K \qquad (5)$$

the values of the four constants being listed in Table 10. The equation means that the survival time of zebrafish in a given concentration of zinc is inversely correlated with their rate of oxygen consumption. Lloyd (1961a) concluded that the survival time of trout in zinc sulphate and several other poisons was inversely correlated with the rate of water flow through the gills, over a wide range of dissolved-oxygen concentrations (Section 2b (iv)). Lloyd's conclusion and mine appear to be compatible because in well aerated water the

rate of oxygen uptake would be proportional to respiratory flow, providing that the efficiency of oxygen utilization remained unchanged. Equation 5 would probably not hold if the dissolved-oxygen concentration were varied, and Lloyd's data obviously do not apply to embryos which have not developed gills. The two conclusions are therefore considered to be complementary.

Survival time and dry weight (T & W) are connected by Equation 14,

$$T = \frac{K}{(4720\ W^{-.15} - 1600)} + 1.3 \qquad (14)$$

the values of K being given in Table 10. The equation means that the survival of zebrafish in a given concentration of zinc is directly correlated with a power of their weight. However, it has been noted that adult fish survived indefinitely in 5 or 10 p.p.m. zinc, although from the theoretical curves in Fig. 28 they were expected to die after about 12 hours' exposure. The explanation may lie in a hypothesis by Lloyd (1962), discussed in Section 2c (v). Lloyd proposed that fish suffer gill damage when the rate at which zinc enters the gills exceeds the rate of transfer of zinc into the blood stream. If the rate of transfer equals or exceeds the rate of uptake, the fish survives. The explanation is

supported by data in Section 7b (i) which showed that the first toxic action of zinc on hatched zebrafish resulted in gill damage. Adult fish which survived about 12 hours' exposure to zinc recovered from its toxic effects and survived indefinitely.

Survival time and concentration of zinc (T & C) have been linked by Ostwald's Equation

$$TC^n = K \qquad (2)$$

and by Wuhrmann's Equation

$$(T - T_S)\ (C - C_S)^n = K \qquad (3)$$

The data of Lloyd (1960) and Sprague (1964) have been fitted to Equations 3 and 2 respectively, and the values of the constants given in Section 2b (i). It is theoretically possible to calculate the values of the four constants in Equation 3 from the data in Section 3, either by solving four simultaneous equations or by graphical analysis. This was not done because the range of test concentrations of zinc (5 to 40 p.p.m.) was narrow, and it would have been necessary to extrapolate the theoretical curve far beyond the observed data. There is clearly no simple relationship between T and C for the zebrafish, because different values of the constants would have to be calculated for each age class of fish.

No correlation was observed between survival time and whole-body zinc uptake. For reasons given in Section 6c, it is expected that survival time would be related to the rate of zinc uptake by the gills, but this parameter was not measured. The parameter is of obvious importance, in view of Lloyd's (1962) hypothesis (Section 2c (v)) and the known toxic action of zinc (Section 7b (i)). It is probable that the phenomenon of antagonism in fish (Section 2b (ii)) also involves gill permeability and the rate of uptake of salts. For all these reasons, the rate of uptake of zinc by fish gills is considered to be a profitable area for further study.

Studies on zebrafish embryos proved conclusively that their high resistance was in no way owing to protection by the chorion (Section 4). On the contrary, embryos with the chorion removed actually survived longer in zinc than embryos with the chorion entire. Supplementary observations demonstrated that the zebrafish chorion was completely permeable to a variety of substances, in apparent contrast to the chorion of most other teleost eggs (Section 4c (iii)). This suggests that the mechanism of osmoregulation in teleost eggs, and their resistance to poisons, may vary widely. Further comparative studies are recommended.

Variations in the resistance of zebrafish to zinc poisoning were associated with differences in the mode of

toxic action, which caused four recognisable effects (Section 7b). They were, firstly, gill damage; secondly, the formation of opaque patches in the tissues; thirdly, tissue shrinkage; and fourthly, liver damage and associated oedema of the gills.

Gill damage, as described in Section 7b(i) and illustrated in Figs. 31 to 33, was always associated with rapid toxic action, that is with low resistance. It appears to have followed the same pattern as gill damage caused by a variety of other poisons, by destruction of the gill epithelium (Section 2c (i)). Studies of zebrafish treated by the PAS reaction (Section 7b (i)) confirmed Lloyd's (1960) observation that zinc sulphate does not cause the gills of fish to become clogged with mucus. Earlier reports to the contrary (not supported by histology) are considered to be unreliable.

The formation of opaque patches in the tissues of embryos (Section 4c (ii); Figs. 13 & 14) proceeded slowly, that is, it was associated with high resistance. This form of toxic action may be compared with similar observations by Jones (1938, 1939c) on tadpoles and planarians (Section 2c (v)). Neither Jones nor I examined the affected animals histologically.

The shrinkage of tissues in embryos exposed to extremely high concentrations of zinc occurred rapidly (Section 7b (iii)

Liver damage and oedema of the gills were associated with high resistance, occurring after the prolonged exposure of juvenile zebrafish to chronically toxic concentrations of zinc (Section 7b (ii)). When Crandall and Goodnight (1963) exposed juvenile guppies to low concentrations of zinc and other poisons, extensive damage occurred in many organs, including the liver but excluding the gills (Section 2c (i)). Crandall and Goodnight considered that the observed histological changes were typical of those expected when an animal is subjected to stress, and that the damage was non-specific. Experimental procedures in the present study were generally similar to those used by Crandall and Goodnight It is considered remarkable that two similar studies should yield such strikingly different results. The differences are a reminder that different species may react to the same stimulus in entirely different ways and that it is a dangerous practice to predict the effect of a certain factor on one species from the results of experiments with another. For that reason, it is not claimed that the conclusions of the present study necessarily have wide general application.

It has been possible to trace changes in the resistance of the zebrafish to zinc poisoning through the life cycle,

and largely to explain the changes in terms of oxygen uptake and body size. It is obvious, however, from observations on the toxic action of zinc, that the subject of zinc toxicity is not one which is capable of a simple general explanation. Much more detailed work on the life cycles of other species is necessary before wide generalizations may validly be made.

## 9. SUMMARY

The literature concerning the toxicity of zinc compounds to aquatic animals is reviewed. (Section 2)

Zebrafish of eleven age groups were exposed to four concentrations of zinc sulphate (5, 10, 20 and 40 p.p.m. Zn) in soft water ($CaCO_3$ 10 p.p.m.) at 25°C. In all four concentrations, newly laid eggs survived the longest time. Survival time decreased with age until hatching on the fourth day. Newly hatched fish (4 to 13 days old) survived the shortest time. Forty-day-old fish and adults (100 days old) survived slightly longer in the two highest concentrations. In the two lowest concentrations, 40-day-old fish survived as long as newly laid eggs, and many adults survived indefinitely. The threshold concentration of zinc was approximately 10 p.p.m. for adults and 1.3 p.p.m. for 40-day-old fish. (Sections 3a & 3b)

Four hypotheses are proposed to correlate resistance with other, readily observable factors.

1. The high resistance of unhatched embryos is owing to protection by the chorion.

2. Resistance is inversely proportional to the rate of oxygen uptake.

3. Resistance is inversely proportional to the rate of whole-body zinc uptake.

4. Variations in resistance are associated with differences in toxic action. (Section 3c)

Data testing the four hypotheses follow.

Unhatched 15 and 25-hour-old embryos with the chorion ruptured survived longer in water containing 20 p.p.m. zinc than did embryos of the same ages with the chorion entire. Forty-two hour embryos with this membrane completely removed survived in water containing 2.5, 5, 10 or 20 p.p.m. zinc at least as long as 42-hour embryos with the chorion entire. The high resistance of unhatched embryos was therefore not owing to protection afforded by the chorion, and Hypothesis 1 is rejected. The presence of the chorion actually lowered resistance, possibly owing to the formation of opaque material enclosed by the chorion of unruptured eggs. It is suggested that the combined obstruction of this material, plus the membrane, may have slowed down some vital process of the embryo, causing its earlier immobilization. (Section 4)

The rate of oxygen uptake of thirteen age groups of zebrafish was measured in zinc-free water at 25°C., hardness 10 p.p.m. $CaCO_3$, under standard conditions. The relationship

between survival time in zinc (T) and the rate of oxygen uptake on a dry weight basis (X) was

$$(T - T_S)\ (X - X_S)^n = K$$

where $T_S$, $X_S$, n and K are constants. Hypothesis 2 is therefore supported in a modified form. Survival time (T) was also shown to be connected to the dry weight of the fish (W)

$$T = \frac{K}{(4720\ W^{-.15} - 1600)} + 1.3$$

where K is a constant. (Section 5)

Juvenile fish (50 to 78 days old) and adults were exposed to soft water (10 p.p.m. $CaCO_3$) containing 20, 2, 0.5 or <0.04 p.p.m. zinc labelled with zinc-65, at 25°C. The uptake of zinc was measured. The survival time of juvenile fish in 20 p.p.m. zinc was measured and their rate of whole-body zinc uptake calculated. There was no relationship between survival time in zinc and the rate of whole-body zinc uptake. Hypothesis 3 is therefore rejected. (Section 6)

Variations in the resistance of zebrafish to zinc poisoning were associated with differences in mode of toxic action. Damage to the gill epithelium of hatched fish was always associated with rapid toxic action, that is with low resistance. The formation of opaque patches in the tissues of

embryos proceeded slowly. Rapid shrinkage of tissues in embryos occurred in very high concentrations of zinc. Liver damage and oedema of the gills were associated with high resistance in juvenile fish. The preceding observations, and probit analysis of survival data, support Hypothesis 4. (Section 7)

The experimental data are discussed in relation to the literature. (Section 8)

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# TOXICITY OF ZINC COMPOUNDS TO AQUATIC ANIMALS, WITH SPECIAL REFERENCE TO FISH

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# TOXICITY OF ZINC COMPOUNDS TO AQUATIC ANIMALS, WITH SPECIAL REFERENCE TO FISH

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ABSTRACT

*The toxicity of zinc compounds to aquatic animals is modified by several environmental factors, particularly the hardness of the dilution water, the dissolved oxygen concentration, and temperature. The resistance of aquatic animals to zinc poisoning varies with species. It is modified by acclimatization, and possibly by age. Survival time is inversely proportional to concentration of zinc. For these reasons concentrations reported as lethal have varied widely.*

*The mode of toxic action of zinc is uncertain. At acutely toxic concentrations it probably kills fish by destroying gill tissues. At chronically toxic levels it may induce stress resulting in death. The action of zinc undoubtedly differs at different concentrations, it varies with life history, and it is non-specific.*

## I. INTRODUCTION

THE toxicity of zinc compounds has been chiefly studied with reference to two groups of animals—mammals and fish. The literature on mammals has been reviewed by Underwood (1956) and by Vallee (1959, 1962), and will not be discussed here.

The early literature on fish has been briefly reviewed by Doudoroff and Katz (1953) and Doudoroff (1957), as part of general accounts of the toxicity of numerous metal salts to fish. Because almost all the important studies concerning zinc have been published in the last five years, a new appraisal of the field is now justified. The present survey of the literature is primarily restricted to the toxicity of zinc compounds to aquatic animals, but the effects of other substances will be discussed where some point relevant to zinc toxicity is thereby illustrated.

There have been numerous studies of the acute toxicity of zinc compounds to aquatic animals, and data from the more accessible references are summarized in Table 1. Concentrations reported to be lethal vary from 330 p.p.m. zinc (Carpenter, 1927), to 0.01 p.p.m. (Affleck, 1952). Apart from the enormous volume of largely repetitive work indicated in Table 1, interest in the toxicity of zinc has proceeded along two main lines. On the one hand, investigators have studied how different factors modify the toxicity of zinc. On the other, they have observed the toxic effects of zinc in an attempt to understand the mode of toxic action of the metal. In consequence, this review falls into two parts.

## II. FACTORS INFLUENCING THE TOXICITY OF ZINC COMPOUNDS

The most important factor influencing whether a given concentration of poison will

TABLE 1

*Survival of aquatic animals in water containing zinc*

| ORIGINAL AUTHOR | SOURCE OF DATA | DILUTION WATER* | TEMP. (°C) | TEST ANIMALS | RESULT* ** |
|---|---|---|---|---|---|
| Abbott, 1924 | Cairns & Scheier, 1957 | Devils Lake, Dakota | ? | small fish | dead after 8 hr in Zn 15 |
| Affleck, 1952 | Table 5 | hatchery, Ca 1.7, Mg 1.0 | 4–9 | rainbow trout eggs | 0% hatched in Zn 0.04 |
| Affleck, 1952 | Table 10 | hatchery, Ca 1.7, Mg 1.0 | 8–12 | rainbow trout fry | 46% survived 28 days in Zn 0.01 |
| Affleck, 1952 | Table 10 | hatchery, Ca 1.7, Mg 1.0 | 8–12 | rainbow trout fry | 98% survived 28 days in Zn 0.003 |
| Affleck, 1952 | Table 11 | hatchery, Ca 1.7, Mg 1.0 | 9–12 | rainbow trout fingerlings | 0% survived 1 day in Zn 0.13 |
| Affleck, 1952 | Table 11 | hatchery, Ca 1.7, Mg 1.0 | 9–12 | brown trout fingerlings | 100% survived 20 days in Zn 0.13 |
| Anderson, 1944 | Table 1 | Lake Erie, Ca 31 | 25 | *Daphnia magna* | 16 hr $LC_{50}$ = Zn 19** |
| Anderson, 1948 | Table 1 | Lake Erie, Ca 31 | 25 | *Daphnia magna* | 64 hr $LC_{50}$ = Zn 0.072 |
| Cairns & Scheier, 1957 | Table 2 | synthetic water Ca 11, Mg 4 | 18 | bluegills | 96 hr $LC_{50}$ = Zn 2.9–3.8 |
| Cairns & Scheier, 1957 | Table 2 | synthetic water Ca 11, Mg 4 | 30 | bluegills | 96 hr $LC_{50}$ = Zn 1.9–3.6 |
| Cairns & Scheier, 1957 | Table 2 | synthetic, Ca 8700, Mg 3000 | 18 | bluegills | 96 hr $LC_{50}$ = Zn 10.1–12.5 |
| Cairns & Scheier, 1957 | Table 2 | synthetic, Ca 8700, Mg 3000 | 30 | bluegills | 96 hr $LC_{50}$ = Zn 10.2–12.3 |
| Carpenter, 1927 | Table E | distilled (?) water | 18 (?) | *Phoxinus phoxinus* (minnows) | mean survival time of 200 min in Zn 330 |
| Ellis, 1937 | P. 430 | hard water | ? | goldfish | few survived 5 days in zinc sulfate 100 |
| Fowler, 1931 | Anderson, 1948 | well water | ? | *Daphnia longispina* | Zn 65 toxic in 15 hr |
| Goodman, 1951 | Table 1 | hard water | ? | rainbow trout fingerlings | 24 hr $LC_{50}$ = Zn 2–6 |
| Goodman, 1951 | Table 3 | hard water | ? | rainbow trout fingerlings | 48 hr $LC_{50}$ = Zn 3–4 |
| Grindley, 1946 | Cairns & Scheier, 1957 | ? | ? | rainbow trout | survival time of 133 min in Zn 25 |
| Hutchinson, 1933 | Anderson, 1948 | pond water (?) | ? | *Daphnia magna* and *D. pulex* | survival time < 5 days in Zn 0.65 |
| Jones, 1938 | Table 3 | tap water, Ca 1 | 14–17 | *Gasterosteus aculeatus* (sticklebacks) | 8.5 day $LC_{50}$ = Zn 0.3 |
| Jones, 1938 | Table 3 | tap water, Ca 1 | 14–17 | *Gasterosteus aculeatus* (sticklebacks) | 108 hr $LC_{50}$ = Zn 0.7 |
| Jones, 1938 | Table 3 | tap water, Ca 1 | 14–17 | *Gasterosteus aculeatus* (sticklebacks) | 6 hr $LC_{50}$ = Zn 20 |
| Jones, 1938 | Table 3 | tap water, Ca 1 | 14–17 | *Gasterosteus aculeatus* (sticklebacks) | 143 min $LC_{50}$ = Zn 200 |
| Jones, 1940a | Table 1 | distilled water | 15–18 | *Polycelis* (planarians) | 48 hr $LC_{50}$ = Zn 12 |

TABLE 1 (continued)

*Survival of aquatic animals in water containing zinc*

| ORIGINAL AUTHOR | SOURCE OF DATA | DILUTION WATER* | TEMP. (°C) | TEST ANIMALS | RESULT* ** |
|---|---|---|---|---|---|
| Jones, 1940b | P. 378 | soft river water, Zn 0.7–1.2, Pb 0.05 | ? | ..... | fish absent from river |
| Lloyd, 1960 | Fig. 1 | diluted well water Ca 4.5, Mg 0.19 | 17.5 | rainbow trout fingerlings | 48 hr $LC_{50}$ = Zn 0.6 |
| Lloyd, 1960 | Fig. 1 | diluted well water Ca 19, Mg 0.79 | 17.5 | rainbow trout fingerlings | 48 hr $LC_{50}$ = Zn 2 |
| Lloyd, 1960 | Fig. 1 | well water, Ca 120, Mg 5 | 17.5 | rainbow trout fingerlings | 48 hr $LC_{50}$ = Zn 4 |
| Mathews, 1904 | Table 2 | distilled water | 19–26 | *Fundulus* eggs | no embryos developed in Zn 40 |
| Mathews, 1904 | Table 2 | distilled water | 19–26 | *Fundulus* eggs | 18% of embryos developed in Zn 27 |
| Naumann, 1934 | Table 2 | hard water | ? | *Daphnia magna* | all dead in Zn 0.01 in 10 days |
| Naumann, 1934 | Table 2 | soft water | ? | *Daphnia magna* | most survived in Zn 0.1 |
| Oshima, 1931 | Doudoroff & Katz, 1953 | distilled water (?) | 20–22 | young eels | 50 hr $LC_0$ = Zn 0.065 |
| Oshima, 1931 | Doudoroff & Katz, 1953 | distilled water (?) | 20–22 | young eels | 20 hr $LC_{50}$ = Zn 6.5 |
| Rushton, 1949 | Doudoroff & Katz, 1953 | tap water | ? | young carp | survival time < 1 day in Zn 0.5 |
| Thomas, 1915 | Doudoroff & Katz, 1953 | sea water | ? | *Fundulus* (marine strain) | zinc sulfate 200 non-toxic |
| Thomas, 1915 | Doudoroff & Katz, 1953 | freshwater | ? | *Fundulus* (freshwater strain) | survival time 2 days in zinc sulfate 10 |
| Wurtz, 1962 | Table 2 | water, $CaCO_3$ 20 | 13 | Young *Physa heterotropha* (snails) | 96 hr $LC_{50}$ = Zn 1.4 |
| Wurtz, 1962 | Table 2 | water, $CaCO_3$ 100 | 13 | Young *Physa heterotropha* (snails) | 96 hr $LC_{50}$ = Zn 0.43 |
| Wurtz, 1962 | Table 3 | water, $CaCO_3$ 20 | 13 | *Helisoma companulata* (snails) | 96 hr $LC_{50}$ = Zn 3.0 |
| Wurtz, 1962 | Table 3 | water, $CaCO_3$ 100 | 13 | *Helisoma companulata* (snails) | 96 hr $LC_{50}$ = Zn 0.87 |

* All concentrations expressed in parts per million.

** "16 hr $LC_{50}$" means "the lethal concentration killing 50% of the test animals in 16 hours." Similarly, "50 hr $LC_0$" means "the highest concentration killing 0% of the test animals in 50 hours."

kill an aquatic animal is the duration of exposure. Toxicity is influenced by environmental factors such as temperature, pH, dissolved oxygen, and carbon dioxide. The toxicity of zinc compounds in particular is modified by compounds of other heavy metals and of the alkaline earths. The complexing of zinc ions with different radicals may further modify its toxicity. The resistance of aquatic animals to a given toxic environment also differs at both the species and the individual levels. It may vary with life history and with prior exposure to the poison or to other environmental factors.

Some of the variation in the lethal concentrations reported in the literature is owing to factors in the bioassay conditions that do not normally apply in nature. Between different investigations, the ratio of biomass of test animals to quantity of available poison has varied widely, at any given concentration. Finally, different workers have selected different physiological criteria to mark the response of the animal that would have terminated in its death.

The importance of those factors influencing the toxicity of zinc compounds will now be discussed.

### *a. Relation between concentration and survival time*

The survival time of aquatic animals exposed to a poison is inversely related to the concentration of poison. There may or may not be a threshold concentration, defined by Bliss (1940) as the highest concentration that would just fail to kill under prolonged (theoretically infinite) exposure. Similarly, there may or may not be a threshold time to death, a threshold that is defined here as the lowest exposure time necessary to produce death under the highest attainable concentration. In practice, the death of an animal is rarely taken as the end point of a toxicity bioassay, but rather some earlier reaction (such as immobilization) that is known to precede death.

There have been three studies of the time-concentration relationship for zinc. The first was by Jones (1938), who exposed juvenile sticklebacks (*Gasterosteus aculeatus*) to zinc sulfate dissolved in extremely soft tap water (1 p.p.m. calcium) at 14 to 17° C. He calculated the arithmetic mean survival time (end point unspecified), and plotted time against concentration (Fig. 1). He then repeated this work with mature sticklebacks (Table 2).

For exposure times greater than two days, the resistance of the two sets of fish appears to have been approximately similar. From Fig. 1, Jones estimated the threshold concentration to be less than 0.3 p.p.m. zinc. The data in Table 2 indicate that the threshold time was less than 109 minutes.

Anderson (1948) exposed *Daphnia magna* to zinc chloride dissolved in soft water from Lake Erie, at a temperature of 25° C. For each group of daphnids exposed to the same concentration of zinc he calculated the geometric mean time to immobilization, which may be defined by the following equation.

$$GMT_i = n\sqrt{T_1 \cdot T_2 \cdot T_3 \ldots T_n} \qquad (1)$$

where $T_1$, $T_2$, $T_3$, . . . $T_n$ are the individual times to immobilization of $n$ animals. Anderson then plotted mean time against concentration,

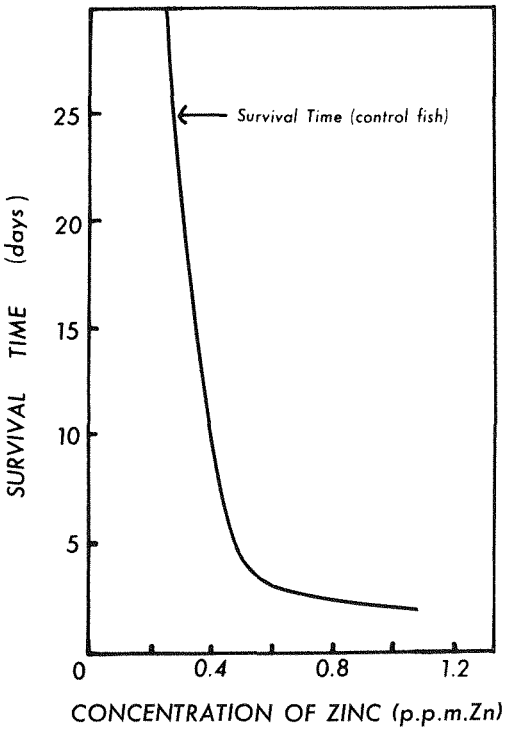


FIG. 1. TOXICITY OF ZINC SULFATE TO JUVENILE STICKLEBACKS (*Gasterosteus aculeatus*) IN SOFT WATER, Ca 1 p.p.m.

After Jones, 1938, Fig. 1.

TABLE 2

*Toxicity of zinc sulfate to mature sticklebacks (Gasterosteus aculeatus) in soft water, Ca 1 p.p.m.*

| CONCENTRATION OF ZINC (PARTS PER MILLION) | MEAN SURVIVAL TIME |
|---|---|
| 300 | 109 min |
| 100 | 207 min |
| 30 | 5.3 hr |
| 10 | 7.8 hr |
| 3.0 | 16.5 hr |
| 1.0 | 34 hr |
| 0.3 | 8.5 days |
| 0.1 | 11.5 days |
| 0.0 | 10.5 days |

(Modified after Jones, 1938, Table 3.)

both axes on a logarithmic scale (Fig. 2). He estimated that the threshold concentration of zinc to *Daphnia magna* was less than 0.072 p.p.m. The shape of the graph (Fig. 2) suggests that there was no threshold time of response. Anderson attributed the irregular shape of the regression line to the variable resistance of daphnids with age. This point will be discussed further in the section on the resistance of aquatic animals.

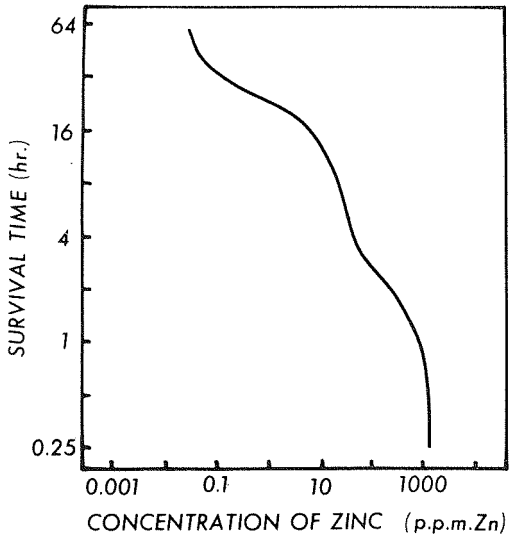


FIG. 2. TOXICITY OF ZINC CHLORIDE TO *Daphnia magna* IN LAKE ERIE WATER, Ca 31 p.p.m.

After Anderson, 1948, Fig. 2.

Lloyd (1960) exposed fingerling rainbow trout (*Salmo gairdneri*) to zinc sulfate dissolved in well water or diluted well water, at three levels of hardness, and at a temperature of 17.5° C. He graphically estimated the median time to immobilization of each batch of fish exposed to identical conditions by the method suggested by Bliss (1937). Lloyd, treating the data from each hardness level separately, then plotted the logarithm of the median time against the logarithm of concentration of zinc (Fig. 3).

Curve C shows that the relationship between the logarithm of concentration of zinc and the logarithm of survival time was linear in soft water, within the range of concentrations examined. Regression lines of this type may be fitted to Ostwald's equation (1907).

$$C^n T = K \qquad (2)$$

where $C$ = concentration, $T$ = time, and $n$ and $K$ are constants. Lloyd's value for $n$ and $K$ were 1.06 and 1650 minutes respectively. As zinc is known to be an essential element in animal metabolism (e.g. Vallee, 1959), it clearly cannot be toxic in minute traces. It follows that a certain threshold concentration is necessary to kill an aquatic animal, and that Curve C cannot therefore extend as far as the Y axis. A theoretically possible continuation of Curve C is shown by means of a dotted line. On the basis of further experimental work, Lloyd (1961b, Fig. 3) decided that the regression line was in fact

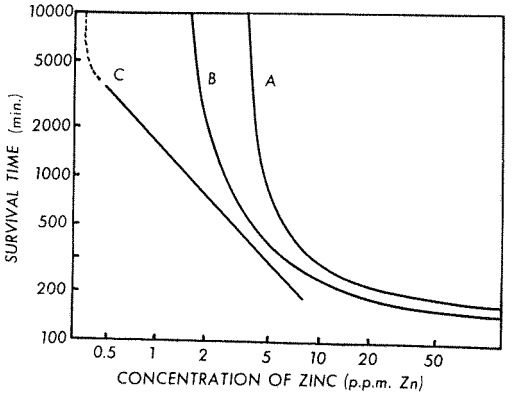


FIG. 3. TOXICITY OF ZINC SULFATE TO RAINBOW TROUT IN WATERS OF DIFFERENT TOTAL HARDNESS

A, total hardness 320 p.p.m. as $CaCO_3$. B, 50 p.p.m. as $CaCO_3$. C, 12 p.p.m. as $CaCO_3$. (After Lloyd, 1960, Fig. 1.)

curvilinear, with a threshold concentration of 0.56 p.p.m. zinc.

The data for hard water indicate a curvilinear relationship between the logarithms of concentration and time (Curves A and B). Lloyd subtracted appropriate constants from concentration and time ($C_S$ and $T_S$ respectively), until the relationship between the logarithms of $(C - C_S)$ and $(T - T_S)$ was linear. The equation of the resulting regression line was developed by Wuhrmann (1952).

$$(C - C_S)^n (T - T_S) = K \qquad (3)$$

Lloyd's values for the four constants $C_S$, $T_S$, $n$, and $K$, to fit his data, were as follows:

| | *Curve A* | *Curve B* |
|---|---|---|
| $C_S$ | 3.5 p.p.m. Zn | 1.5 p.p.m. Zn |
| $T_S$ | 160 min | 140 min |
| $n$ | 1.24 | 1.43 |
| $K$ | 813 min | 933 min |

Both Lloyd and Wuhrmann assumed that the mathematical constants $C_S$ and $T_S$ indicated threshold concentration and threshold time, respectively.

In all the data presented so far, the zinc was probably in the form of zinc ions ($Zn^{++}$). To test whether suspended zinc (possibly $ZnCO_3$) was also toxic, Lloyd (1960) exposed batches of trout to three treatments. In the first, there was no suspended zinc; in the second, there were 9 p.p.m. suspended zinc; and in the third, 18.5 p.p.m. There were approximately 11 p.p.m. zinc in solution in each case. Survival time was 285 minutes in the first treatment, 180 minutes in the second, and only 162 minutes in the third. The suspended zinc thus reduced survival time substantially.

The toxicity of soluble complex compounds containing zinc has been considered only by Doudoroff (1956). He found that the toxicity of the zinc cyanide ion ($Zn(CN)_4^{--}$) to *Pimephales promelas* (a minnow) was greater than could be predicted from that of the zinc ion and cyanide ion taken separately. For example, half the fish were killed in 96 hours by either 0.23 p.p.m. sodium cyanide (as CN) or 0.18 p.p.m. sodium cyanide (as CN) plus 0.125 p.p.m. zinc sulfate (as Zn). One part per million zinc sulfate (as Zn) alone did not kill any fish. Doudoroff's results therefore suggest synergism, as defined below (sect. IIc).

The toxicity of complex compounds involving heavy metals apparently shows no consistent pattern. Doudoroff (1956) estimated the toxicity of cadmium cyanide to be greater, and the toxicities of copper and nickel cyanides to be less, than would be predicted from the toxicities of the appropriate ions taken separately. The only other comparable study has been by Jones (1940c), who concluded that the double chlorides of mercury and sodium were less toxic than would be expected from the toxicities of mercury, sodium, and chloride ions taken separately.

### *b. Effect of salts of the alkaline-earth metals*

Lloyd (1960) considered hardness to be the most important single factor modifying the toxicity of zinc ions. He measured the survival time of rainbow trout in a series of concentrations of zinc, at three hardness levels. His methods have been described above (sect. IIa) and his results were presented in Fig. 3. The hardness of the three dilution waters was made up as follows:

| CURVE | TOTAL HARDNESS (p.p.m. $CaCO_3$) | TOTAL CALCIUM (p.p.m. Ca) | TOTAL MAGNESIUM (p.p.m. Mg) |
|---|---|---|---|
| A | 320 | 120 | 5 |
| B | 50 | 19 | 0.79 |
| C | 12 | 4.5 | 0.19 |

In each case, 94 per cent of the hardness was due to calcium ions.

Lloyd observed that the effect of hardness increased with increase in period of survival, until there was a ten-fold difference between the toxicities of zinc in the hardest and softest water over $2\frac{1}{2}$ days' exposure. As the curves were of different shapes, Lloyd deduced that the ratio between the toxicities of zinc at any two hardness levels was not constant. However, he has recently modified this opinion, and in the light of new evidence he has postulated a linear relationship between the logarithms of threshold concentration of zinc and total hardness respectively (Department of Scientific and Industrial Research, 1961, Fig. 54).

The remaining data on the toxicity of zinc in hard and soft water are of little more than historical interest. Jones (1938) observed that

sticklebacks survived for ten days in water containing 2 p.p.m. zinc and 50 p.p.m. calcium, but died in 2 p.p.m. zinc and no calcium. Cairns and Scheier (1957) estimated that the concentration of zinc killing half a sample of bluegills (*Lepomis macrochirus*) in four days was 11.3 p.p.m. when the hardness was approximately 30,000 p.p.m. calcium carbonate, but only 2.8 when the hardness was approximately 40 p.p.m. calcium carbonate. Only Naumann (1934) has reported zinc to be less toxic in soft water than in hard water. His data are summarized in Table 1.

It has been known since the work of Ringer (1897) that the toxicity of some metal cations to aquatic organisms is reduced in the presence of other metal cations, notably calcium. This phenomenon is known as antagonism, and the literature on it is extensive. The more important reviews are by Heilbrunn (1937), Jones (1939b), and Doudoroff and Katz (1953).

Except in the study by Naumann (1934), antagonism has been detected in every case where the toxicity of heavy metal ions has been observed in the presence of ions of the alkaline earths. By far the most important contribution on antagonism involving the heavy metals has been made by Jones (1939a), who studied the toxicity of copper nitrate to tadpoles of *Bufo bufo,* in the presence of the nitrates of either calcium, magnesium, strontium, or barium. At the two levels of copper investigated, Jones found that tadpoles survived longest when the concentration of the alkaline earths was approximately 0.1 normal. With 320 p.p.m. copper present, strontium caused the greatest antagonism, followed by calcium, magnesium, and barium, in that order. With 64 p.p.m. copper present, calcium exerted a greater influence than strontium, followed as before by magnesium and barium. Antagonism was also demonstrated between strontium and nickel ions, in a range of concentrations of both. In contrast, the toxicities of lead nitrate and cadmium nitrate were not reduced by salts of the alkaline earths.

A major criticism of Jones' work is that he only studied the effects of highly toxic solutions of heavy-metal ions, although he himself stated (Jones, 1939a) that the mode of toxic action of the heavy metals undoubtedly varies with concentration. This deficiency has been to some extent filled by the work of Lloyd (1960), who recorded in effect the antagonism of three concentrations of calcium to concentrations of zinc ranging from 0.5 to 50 p.p.m. Unfortunately, no author since Jones has recompared the antagonistic effects of the four alkaline-earth metals, or has remeasured at what concentration these effects reach a maximum.

There are two theories about the mechanism of antagonism by the alkaline-earth metals, and the relevant literature has been briefly reviewed by Jones (1939b). The permeability theory is attributed by Jones to work by Loeb and Osterhout, dating from 1912 and 1914 respectively. The original papers may be traced through a later book by Osterhout (1922). The alternative theory of contrary action was developed by Heilbrunn (1928, 1937).

According to the permeability theory, one compound antagonizes another by reducing the permeability of cell membranes, and thereby reducing the speed of penetration of the second compound into the tissues. Using a variety of living material, and different methods, Loeb and Osterhout were able to demonstrate that the permeability of sodium and potassium ions into tissues was reduced by the antagonism of ions of the alkaline earths and, to a lesser extent, of the heavy metals.

The antagonism of the alkaline earths to toxic solutions of zinc may also be owing to a reduction in the permeability of surface membranes (Department of Scientific and Industrial Research, 1958, Figs. 32 and 33). Trout reared in hard water, but exposed to a toxic concentration of zinc in soft water, survived as long as trout reared in either hard or soft water and exposed to zinc in hard water. In all three cases survival time was approximately the same, and was much longer than the survival times of trout that were first reared in soft water and then exposed to zinc in soft water. As the fish that were reared in soft water and tested in hard water had no time in which to absorb calcium or magnesium ions, prior to their exposure to zinc, it is suggested that the antagonism of the zinc ions by the hard water acted at the surface of the fish. This question could perhaps be settled using a convenient radioisotope, such as calcium-45.

The theory of contrary action is not concerned with the penetration of ions through

membranes. Heilbrunn suggested that the toxicity of most metal ions was due to their ability to coagulate protoplasm inside the cell, but that coagulation was inhibited by calcium ions. To date, no one has tested this theory histologically, but it has been observed that fish mucus became coagulated by zinc or lead ions, both on the skin (Carpenter, 1927; Jones, 1938) and in vitro (Jones, 1938). In both cases, coagulation was inhibited if sufficient calcium ions were present. On the other hand, there is slight evidence that salts of the heavy metals are not general internal poisons to fish. Schweiger (1957) injected substantial amounts of either manganese or cadmium salts into the body cavity and alimentary canal of carp, without apparent damage. When Saiki and Mori (1955) injected a trace of zinc-65 into carp muscle, 56 per cent of it was lost from the body within 45 hours.

It is clear that the mechanism of antagonism, concerning both zinc and other heavy metals, is poorly understood. The two theories about antagonism do not appear to be mutually exclusive, and further evidence is needed to evaluate them.

### *c. Effect of salts of the heavy metals*

If an aquatic animal is exposed simultaneously to two poisons, the result may either be predictable from the toxicity of each poison taken separately (additive effect), be greater than predicted (synergism), or be less than predicted (antagonism). Under different conditions, mixtures of zinc ions and the ions of other heavy metals have been shown to demonstrate the first two effects, which will be illustrated here. Antagonism has already been discussed (sect. IIb).

The studies of Bandt (1946) and Doudoroff (1952) have been reviewed by Doudoroff and Katz (1953). Bandt exposed trout and roach to mixtures of sulfates of the heavy metals in soft tap water. His results were as follows:

| | |
|---|---|
| zinc + cadmium | additive effect |
| zinc + nickel | synergistic effect |
| zinc + copper | strongly synergistic, up to 5 times more toxic |

Doudoroff (1952) exposed minnows (*Pimephales*), for eight hours, to solutions containing ions of zinc or copper or a mixture of the two, in soft water. Those fish in either 8 p.p.m. zinc or 0.2 p.p.m. copper survived: others in 1 p.p.m. zinc plus 0.025 p.p.m. copper died. Thus Doudoroff and Bandt both observed pronounced synergism between zinc and copper in soft water.

Lloyd (1961b) exposed rainbow trout to solutions of zinc sulfate, copper sulfate, and a mixture of the two, made up in hard water (320 p.p.m. $CaCO_3$) and soft water (15–20 p.p.m. $CaCO_3$). The mixture was always in the ratio of six parts of zinc to one of copper, by weight. About six concentrations of each of the six series of treatments were tested. In soft water, the experiment was terminated after seven days. The concentration of zinc that immobilized half the trout in that time was 10.5 times the concentration of copper. Lloyd assumed that the same ratio held over all exposure times less than seven days, and drew by eye the best curve of survival time upon concentration of poison that satisfied both the zinc and the copper data. He then fitted the data for the zinc-copper mixture about the same curve. Data falling below the line indicated synergism, because survival time was lower than predicted. This occurred where the concentration of zinc exceeded about 1.8 p.p.m. and copper 0.3 p.p.m. The effects of the zinc and copper at lower concentrations were additive. Lloyd treated the data from the experiments using hard water by the same method, and found that the effects of zinc and copper were additive at all concentrations; but the most toxic mixture only contained about 6 p.p.m. zinc plus 1 p.p.m. copper.

### *d. Effect of dissolved oxygen*

Lloyd (1960) exposed rainbow trout to five lethal concentrations of zinc sulfate at three non-lethal concentrations of dissolved oxygen each, in hard water ($CaCO_3$ 320 p.p.m.), using the methods already described (sect. IIa). He calculated that, over an exposure period of 1000 minutes, the concentration of zinc necessary to kill half the fish was 1.4 times greater at an oxygen concentration of 8.9 p.p.m. than it was at 3.8 p.p.m.

Westfall (1945) found that lead was more toxic to goldfish in water low in oxygen, and

Wuhrmann (1952) observed that ammonia, cyanides, and phenols have all been reported to be more toxic to fish in water low in oxygen. Lloyd (1961a) considered the effect of oxygen concentration on the toxicity to rainbow trout of poisons in general. He illustrated his discussion with examples from his own work on zinc sulfate (described above), lead nitrate, copper sulfate, and a mixture of phenols.

Lloyd measured the concentration ($X_s$) of each poison, that killed half the trout in approximately 1500 minutes, with an oxygen concentration equal to 100 per cent of the air-saturation value. He divided this by the concentration ($X$) of each poison, that also killed half the trout in 1500 minutes, but at a lower concentration of oxygen. Different values for $X_s/X$ were then plotted against the corresponding concentrations of oxygen, expressed as percentage of air-saturation. For all four poisons, the value of $X_s/X$ increased from 1.0 at 100 per cent air-saturation to 1.4 at 40 per cent air-saturation. In further work on the toxicity of ammonium chloride, Lloyd found that $X_s/X$ increased to 2.2 at 40 per cent air-saturation, based on an exposure time of 500 minutes. He was able to demonstrate that this high value of $X_s/X$ was caused by an increase in pH at the gill epithelium, which was caused by a reduction in concentration of excreted carbon dioxide, which was in turn associated with a drop in oxygen consumption.

As $X_s/X$ is similar for different poisons (under otherwise identical conditions), Lloyd suggested that low oxygen concentration causes a physiological response independent of the effect of the poison. The most obvious reaction of fish under stress is an increase in the rate of opercular movement. If it be assumed that the amplitude of opercular movement remained unchanged, the rate of flow of water over the gills would then be increased, which in turn would increase the amount of poison reaching the gill epithelium. Lloyd assumed that this is the site where most poisons are absorbed by fish. He cited the work of Weiss and Botts (1957) as showing that the toxicity of an organic poison to several species of fish was increased by either an increase in oxygen uptake or by low oxygen concentration. In both cases, Weiss and Botts thought that the toxicity of the poison was proportional to the rate of respiratory flow. Lloyd then argued, on theoretical grounds, that this conclusion was justified.

If Lloyd is correct in concluding that the survival time of a fish in a toxic solution is proportional to the rate of flow of water over its gills, then it follows that any environmental change that causes a change in respiratory flow will have the same effect as a change in oxygen tension. Lloyd based his hypothesis on a comparison of concentrations of five poisons under only three levels of dissolved oxygen, and under only one level each of temperature, water hardness, and exposure time. The importance of the theory would justify a much more extensive study of a number of the variables influencing toxicity.

### *e. Effect of carbon dioxide*

There is no direct evidence about the effect of carbon dioxide concentration on the toxicity of zinc, and only scanty indirect data. Lloyd (1960) compared the survival times of rainbow trout in five concentrations of zinc, made up in two batches of hard water (hardness probably equivalent to 320 p.p.m. $CaCO_3$). The hardness in the first batch was due mainly to calcium bicarbonate, and in the second mainly to calcium chloride. Survival times in the bicarbonate solutions were consistently lower. Presumably this difference was owing to the toxicity of either bicarbonate ions or carbon dioxide, or a combination of the two, but the relative concentrations of each cannot be deduced from Lloyd's data.

### *f. Effect of temperature*

There have been two studies of the effect of temperature on zinc toxicity. Lloyd (1960) compared the survival times of rainbow trout in four concentrations of zinc, in hard water, tested at four temperature levels. Fish were held for five days and then tested at temperatures of 13.5, 15.5, 18.5, or 21.5° C. Survival times were generally lower in the warmer water, but the threshold concentration appears to be unchanged. Lloyd calculated that a rise in temperature from 12 to 22° C reduced the sur-

vival time in relatively high concentrations of zinc by a factor of 2.35. Presumably this factor would not hold at concentrations approaching the threshold.

In apparent contrast to Lloyd, Cairns and Schcier (1957) observed little difference between the toxicity of zinc at 18 and 30° C, in hard or soft water, to bluegills held for several days at test temperatures and test hardnesses. However, the two results are not necessarily contradictory, because Cairns and Scheier were concerned with long exposure periods (24 hours or more). It is probable, therefore, that the concentrations they reported as lethal were relatively close to threshold values.

### *g. Resistance of aquatic animals*

Data from Table 1 indicate that widely different concentrations of zinc compounds have been reported as toxic to different species of aquatic animals. It is not possible, however, to list the different species in order of their resistance to zinc, because bioassay conditions have differed greatly. No author has effectively compared the relative resistance of any two species of aquatic animals to zinc poisoning. This information cannot be deduced from the known resistance of aquatic animals to other poisons because a species that is more resistant than another to one poison may be less resistant to a second poison (e.g., Wuhrmann, 1952; Applegate, Howell, Hall, and Smith, 1957).

Resistance to poisons also varies greatly between individuals of the same species. Lloyd (1960) found that the logarithms of individual survival times of trout in a given concentration of zinc were normally distributed. Because of this, he was able to estimate the median results for his data graphically, by plotting the logarithms of survival time against probit kill (see sect. IIa). Weiss and Botts (1957) found that larger fish tended to be more resistant to an organic poison than smaller fish of the same species, and that the resistance appeared to be related to oxygen consumption. The latter point is referred to further in sect. IId.

The resistance of a population of animals to a poison may vary as a result of at least three factors: acclimatization (or adaptation) to the poison or some other environmental factor, development of a new phase in the life history, and survival of a resistant group by selective mortality. Several authors have discussed one or more of these factors with reference to zinc. Each factor will now be considered in turn, beginning with data indicating acclimatization.

Lloyd (1960) exposed two batches of rainbow trout to 3.5 p.p.m. and 2.5 p.p.m. zinc respectively, in hard water, for fourteen days. At the end of this period, the fish were transferred to 10 p.p.m. zinc, together with a control group. The fish previously held in 3.5 p.p.m. zinc survived 500 minutes, those held in 2.5 p.p.m. survived 400 minutes, and the control group only 290 minutes. In another experiment, Lloyd held three batches of trout in 9.45, 6.3, and 3.65 p.p.m. of dissolved oxygen for eighteen hours. He then exposed subsamples of each batch to five lethal concentrations of zinc in hard water. Fish held in water low in oxygen, and exposed to zinc at the same oxygen tension, survived longer than fish not acclimatized to low oxygen tension. Finally, it has already been mentioned (sect. IIb) that trout held by Lloyd in hard water, and then exposed to a lethal concentration of zinc in soft water, survived longer than trout held and tested in soft water (D.S.I.R., 1958). Lloyd was thus able to demonstrate that the resistance of rainbow trout to zinc poisoning was raised if the fish became acclimatized to either a sublethal concentration of zinc, or to a low concentration of dissolved oxygen, or to hard water.

Observations on the resistance to zinc poisoning of test animals of different ages have been noted by Jones (1938) and by Anderson (1948). Jones' study of the toxicity of zinc sulfate to sticklebacks has already been summarized (sect. IIa). Jones found that the resistance of juveniles (lengths 18 to 20 mm) and sexually mature adults (lengths 45–50 mm) was approximately similar throughout the range of concentrations of zinc tested. Anderson's work on the toxicity of zinc chloride to daphnids is also discussed in sect. IIa. Anderson attributed the irregular shape of the regression line in Fig. 2 to variations in the resistance of the test animals. He cited an earlier study (Anderson and Jenkins, 1942) showing that, at 25° C, *Daphnia magna* molts at about twenty hours after release from

its mother. A reduction in slope of the regression line at about this time indicates a reduction in resistance of the test animals. Anderson suggested that *Daphnia magna* is less resistant to zinc poisoning at ecdysis because penetration of the animal by zinc ions is then easier.

An increase in resistance of a population of aquatic animals to any poison by selective mortality alone has not yet been reported, either in the laboratory or the field, although the phenomenon is well known among insects exposed to insecticides and microorganisms exposed to antibiotics (e.g., Brown, 1960; Miller and Bohnhoff, 1950). However, Paul (1952) has described an interesting situation in California, where the concentration of copper in certain polluted streams was high enough to kill introduced, hatchery-reared fish, although the resident fish population was apparently unharmed. Paul attributed the resistance of the native fish to acclimatization, but from the meager data selective mortality seems equally possible.

The only other reports indicating variable resistance to zinc poisoning are by Goodman (1951) and Affleck (1952). Goodman maintained fry of rainbow trout in tap water containing 1 p.p.m. zinc. Samples of twenty fry of known ages were then exposed to a range of concentrations of zinc for two days. A selection of Goodman's results is given in Table 3.

Goodman concluded that the resistance of trout fry to zinc increased with age. Affleck found that trout fry previously held in water contaminated with an unspecified concentration of zinc survived a subsequent exposure to a low concentration of zinc better than fry of the same age reared in zinc-free water. Affleck attributed this increased resistance to acclimatization.

In both studies, the mortality of trout fry during the rearing period was unspecified. Therefore, in either case, the observed differences in resistance may have been due to selective mortality of the fry during rearing caused by low concentrations of zinc, or to increasing acclimatization. With Goodman's data, there is the third possibility that resistance may have increased with age. Because of this uncertainty, the conclusions of both authors must be treated with caution.

TABLE 3

*Survival of rainbow trout (Salmo gairdneri) following exposure to solutions of zinc sulfate for two days*

| AGE OF FRY (weeks) | CONC. OF ZINC (p.p.m.) | NO. OF SURVIVORS (in samples of 20) |
|---|---|---|
| 2 | 3 | 11 |
| 2 | 4 | 2 |
| 4 | 4 | 2 |
| 8 | 4 | 18 |
| 10 | 4 | 20 |

(Data from Goodman, 1951.)

### *h. Other factors*

The remaining factors that may modify the reported toxicity of zinc result from limitations of the toxicity bioassay. The number and size of animals in the test solutions will be considered first.

The absorption or precipitation of dissolved zinc by aquatic animals has been demonstrated by Jones (1938), Saiki and Mori (1955), Joyner (1961), and several other workers. It follows that when animals are exposed to zinc in a limited volume of solution, the concentration of zinc will be reduced by the animals. If the biomass of the animals is large, and the volume of solution is small, the concentration of zinc may be substantially reduced, and the survival time of the animals in a lethal concentration will then be unnaturally high. In a toxicity bioassay, the importance of minimizing the ratio of biomass of test animals to mass of available poison is thus apparent.

The effect of high ratios of mass of animals to mass of poison has been demonstrated by Carpenter (1927, 1930) in her work on the toxicity of lead nitrate to minnows. She found that when a small sample of solution was assayed several times using a series of fresh fish, each fish survived longer than the previous one. In another series of experiments, survival of fish in small samples of toxic solution was compared with the survival of similar fish in larger samples of the same concentrations. The smallest concentration of lead that produced the maximum toxic effect was 6200 p.p.m., using 1.8 grams of

fish in half a liter of solution (Carpenter, 1927, p. 383). From these figures it can be calculated that the critical ratio of biomass of fish to mass of available lead was 0.6 to 1. To maintain this ratio using a solution of 1 p.p.m. lead, and one fish weighing one gram, one would require a volume of not less than 1700 liters! Satisfactory ratios may be achieved more conveniently by using very small test animals, or by continually replacing the solution.

The critical ratio of test animals to available zinc has not been determined. Most of the published work on the toxicity of zinc does not include the weight of the test animals, so that the ratios cannot usually be calculated. Jones (1938), Goodman (1951), Cairns and Scheier (1957), and Lloyd (1960) all appear to have used fish : zinc ratios of at least 500 : 1. A notable exception is Anderson (1948), who achieved a ratio of about 10 : 1.

Another factor that may partly explain the wide range of zinc concentrations reported as lethal is the choice of end point. Different investigators have selected a variety of responses to mark the reaction of test animals to a toxic environment. The subject has been discussed by Wuhrmann (1952), who listed the following possible end points to a toxicity bioassay:

1. initial response to toxic action (such as increased ventilation rate);
2. manifestation time, or overturn time;
3. lethal time, or the onset of irreversible change;
4. death.

Of the four, Wuhrmann considered overturn time to be the most precise and the easiest to ascertain. However, most investigators of zinc toxicity have chosen some aspect of death as the end point. Carpenter (1927) selected total immobilization, and Anderson (1948) the cessation of swimming. Cairns and Scheier (1957) and Lloyd (1960) all established immobilization of the gills as the end point. Most other workers terminated the bioassays at death, without defining how death was determined.

Finally, an irritating amount of ambiguity in the literature has arisen from authors' failure to state the chemical formulas on which their lethal concentrations were based, and from doubts about the purity of their compounds. Doudoroff and Katz (1953, p. 817–18) have discussed these objections.

## III. TOXIC ACTION OF ZINC SALTS ON AQUATIC ANIMALS

The factors known to influence the toxicity of zinc salts to aquatic animals have been enumerated. The toxic action of zinc will now be considered, beginning with a survey of the morphological and physiological changes caused by zinc. The entry into, and accumulation of, zinc in the body will then be discussed. Finally, theories concerning the toxic action of zinc will be evaluated.

### *a. Changes in morphology*

It is convenient to describe separately the effects of acutely toxic and chronically toxic concentrations of zinc, because exposure to different concentrations produces different results. Early observations on morphological changes in aquatic animals were limited to changes caused by acutely toxic concentrations, and this work will be considered first.

Carpenter (1927) and Jones (1938) reported gill damage and copious secretions of mucus in minnows (*Phoxinus phoxinus*) and sticklebacks (*Gasterosteus aculeatus*) killed by high concentrations of zinc. The mucus was believed to be the major cause of death, through mechanical obstruction of the gills. Carpenter showed that the mucus probably contained zinc because it turned brown when treated with ammonium sulfide. Jones demonstrated that a toxic solution of zinc sulfate precipitated filtered eel mucus, but neither the clogging of the gills nor cellular damage was apparently confirmed histologically by either author, and it is believed that this may be a crucial shortcoming of their evaluations.

Lloyd (1960) briefly reported the results of a histological study, made for him by Dr. Gwyneth Parry, on the gills of trout that had been exposed to different concentrations of zinc. In 20 p.p.m. zinc, cytological breakdown of the gill epithelium occurred within $2\frac{1}{2}$ hours, but it is not clear whether or not the fish had overturned. In 4 p.p.m. zinc, the gill lamellae became swollen before death. No change was

detected in fish exposed to 3 p.p.m. zinc for two days.

Some additional information about Lloyd and Parry's work is supplied by the report of the Department of Scientific and Industrial Research (1960, p. 83). Unspecified but toxic concentrations of zinc, lead, and copper salts all acted on the gills of trout as follows. After half the expected survival time had elapsed, the epithelium began to separate from the filaments and lamellae. By the time the fish had overturned, approximately half the epithelium had been sloughed off, and at death, three-quarters. Thus, at death, three-quarters of the gills apparently consisted of an undamaged network of blood capillaries from which the gill epithelium had been removed. It is not stated whether the sloughed-off cells clogged the intact gill tissue, or whether they became detached.

Lloyd (1960) reported further that Parry rarely observed any precipitated mucus in the gill chamber at any concentration of zinc, but no special staining technique for mucus (e.g., the periodic-Schiff reaction) appears to have been employed. Using a radioisotope of zinc ($Zn^{65}$), Lloyd detected zinc in mucus secreted over the body surface of zinc-immobilized fish, but he did not see any coagulated mucus on the gills. On the basis of his and Parry's work, Lloyd considered that mucus did not clog the gills of fish that were killed by zinc.

The only published information on morphological changes in aquatic animals caused by chronically toxic concentrations of zinc salts is contained in two careful studies by Crandall and Goodnight (1962, 1963). These authors (1962) exposed 79 new-born guppies (*Lebistes reticulatus*) to 1.15 p.p.m. zinc, in tap water (hardness 80 p.p.m. $CaCO_3$) at 25 to 27° C. The ratio of fish to available zinc was not stated, but was probably in the order of 20,000 : 1. It is therefore possible that the fish were exposed to much lower concentrations of zinc than the authors believed. It is significant that Cairns and Scheier (1957) found that fish exposed to 1 p.p.m. zinc were able to precipitate most of it as mucus within 24 hours.

Crandall and Goodnight found that the 79 guppies reared in zinc solution grew less rapidly than 54 similar fish in zinc-free water. The experimental group had a higher mortality rate and showed less sexual dimorphism. For example, after 90 days the median weights of the experimental and control groups were 23 mg and 52 mg respectively. Similarly, the cumulative mortality was 41 per cent compared with 9 per cent. Only one experimental fish out of 79 developed a gonopodium (male reproductive organ), compared with 30 to 40 per cent of the control fish. In a further experiment, the mortality of 22 guppies exposed to 2.3 p.p.m. zinc was 60 per cent in from 49 to 69 days, at which point the study was terminated.

In their second study (1963), Crandall and Goodnight again exposed new-born guppies to either 1.15 or 2.3 p.p.m. zinc, under the same conditions as before. Live fish were removed periodically, preserved, sectioned longitudinally, stained with hematoxylin and eosin, and examined histologically. After 55 to 65 days in 1.15 p.p.m. zinc, blood vessels in the liver were poorly developed, the mesenteries were practically devoid of fat, the kidney tubules and glomeruli were distended, the lymphoid tissue in the kidneys was reduced, and the gonads were underdeveloped. After 95 days, the liver contained large vacuoles, and granulocytes had accumulated in the heart muscle. The kidney tubules were even more expanded, the spleen was underdeveloped, and only one-quarter of the fish were sexually mature. Control fish of the same age were all sexually mature and showed no abnormalities.

After 58 to 70 days in 2.3 p.p.m. zinc, the liver had degenerated and contained large vacuoles and irregular nuclei. The mesenteries contained no fat, the pancreas was undersized, the kidneys were distorted and hemorrhaged, and the skeletal muscles were underdeveloped and vacuolated. In none of the fish examined was there any gill damage.

The available information indicates that rapidly lethal concentrations of zinc definitely cause severe cytological damage to the gills and some coagulation of mucus over large areas of the body. Opinion is divided as to whether coagulation in the gill cavity is severe enough to clog the gills. No other morphological changes have been observed. Chronically toxic concentrations of zinc, on the other hand, cause no damage to the gills, but induce extensive

deterioration to liver, kidneys, heart, skeletal muscles, gonads, and spleen.

Studies by several authors concerning the effects of different poisons on a variety of fish indicate that none of the morphological changes just described are peculiar to zinc poisoning. Gill damage, sometimes accompanied by coagulated mucus on the gills, has been reported in fish exposed to rapidly lethal concentrations of lead salts (Carpenter, 1927; Ellis, 1937; Jones, 1938, 1939c; Lloyd, 1960), copper salts (D.S.I.R., 1960), salts of various other heavy metals (Schweiger, 1957), trifluoromethyl nitrophenol (Christie and Battle, 1963), and dodecylbenzene sulfonate (Schmid and Mann, 1961, 1962). Observations of gill damage, in the last four papers mentioned, were supported by histological data. The significance of gill damage caused by fish poisons will be discussed below.

The effects on guppies of chronically toxic concentrations of lead nitrate and sodium pentachlorophenate was also reported by Crandall and Goodnight in their two papers already cited. In addition, Dawson (1935) studied the effects of low concentrations of lead acetate on catfish (*Ameiurus nebulosus*). With all three poisons, generally similar morphological changes occurred to those caused by chronic zinc poisoning. The significance of these results will also be discussed in sect. IIIe.

### *b. Changes in physiology and behavior*

Jones (1938) observed that the rate of opercular movements of three-spined sticklebacks (*Gasterosteus aculeatus*) increased when the fish were exposed to an acutely toxic concentration of zinc sulfate. In tap water, the opercular rate was approximately 100 beats per minute. When the fish were introduced into a toxic solution of zinc sulfate (either 2 p.p.m. or 10 p.p.m. zinc) the rate increased steadily to 240 beats per minute and then declined rapidly until death. Naturally, the response of fish in the higher concentration was quicker. Fish removed from the toxic solutions, while the opercular rate was 240 per minute, recovered, and the rate returned to normal. Increases in opercular rates have also been recorded in fish exposed to acutely toxic concentrations of lead nitrate, copper sulfate, and mercuric chloride (Jones, 1938; Carpenter, 1927).

In a later study, Jones (1947a) was able to demonstrate that an increase in opercular rate coincided with a decrease in oxygen consumption by sticklebacks exposed to lethal concentrations of lead, copper, and mercuric salts. Carpenter (1927) made a parallel observation that carbon dioxide production decreased as opercular rate increased when sticklebacks were exposed to a toxic solution of lead nitrate. Thus in both studies the rate of gill movements increased as the rate of gas transfer decreased. However, when Jones exposed sticklebacks to toxic solutions of cyanide and sulfide, oxygen consumption and opercular rate both decreased.

Jones explained this difference in response as follows. Cyanides and sulfides inhibit tissue respiration. The carbon dioxide concentration of the blood is therefore lowered, causing both gill movements and gas exchange to decline. Heavy-metal salts, in contrast, do not significantly inhibit tissue respiration. As gas exchange becomes increasingly prevented at the gill surface, so the carbon dioxide content of the blood rises. The respiratory center is then stimulated, and causes the opercular movements to increase in rate and amplitude.

It follows from Jones' argument that the drop in oxygen consumption upon exposure to heavy-metal salts is probably not caused by a reduced flow of water through the gills but by the reduced efficiency of the gills (owing perhaps to gill damage, circulatory changes, or coagulated mucus). Unfortunately, no information is available on any of these three points, with the exception of an interesting observation by Ellis (1937).

Ellis studied the rate of heart-beat of the carp, by inserting a needle into the pericardial cavity until the point just touched the ventricle of the heart. As the heart continued to beat, so the visible end of the needle oscillated. Ellis observed that when a carp was exposed to a solution of the salt of a heavy metal, the heart-beat remained strong while the gills gradually became clogged with mucus — and presumably damaged cytologically. At some critical point, the heart suddenly began to beat at about half its former rate. Ellis claims to have demonstrated that blood capillaries on the afferent

(cardiac) side of the gills became gorged with blood, while on the efferent side the blood flow almost ceased. He concluded that the mucus prevented contraction of the gill filaments, this being necessary to maintain normal blood circulation. In the complete absence of histological data, this inference must be considered inconclusive.

The behavior of the ten-spine stickleback (*Pygosteus pungitius*), when exposed to lethal concentrations of zinc sulfate and other fish poisons, has been studied by Jones (1947b). The fish were held in a cylindrical, horizontal glass tube, entirely filled with fluid. One half of the tube contained a toxic solution, and the other half contained tap water. The fish were free to swim anywhere in the apparatus, but they avoided concentrations of zinc ranging from 1300 p.p.m. down to 10 p.p.m. by swimming to the other end of the tube. The avoidance reaction took longer at lower concentrations. In a further experiment, fish detected and avoided a concentration of 330 p.p.m. zinc, even after being held forcibly in that concentration for 36 minutes.

Sticklebacks also avoided lethal concentrations of mercuric chloride, ethyl alcohol, formalin, and chloroform. They avoided 3,200 p.p.m. copper (as sulfate), but apparently could not detect 32 p.p.m. and subsequently succumbed to it. Copper sulfate also appeared to destroy the fish's ability to detect zinc sulfate and other poisons, because the usual avoidance reaction did not occur in mixed solutions containing copper sulfate.

All the observations reported so far in this section were on fish held in acutely toxic concentrations of zinc or other poisons. Crandall and Goodnight (1962) are the only workers to have commented on physiological or behavioral changes in fish exposed to chronic concentrations of zinc. In 1.15 p.p.m. zinc, under conditions described in the preceding section, guppies were less active than the controls, ate less, swam abnormally, and had difficulty in maintaining equilibrium. The same general observations were made on guppies held in lead nitrate and sodium pentachlorophenate solutions, except that these fish had normal appetites.

*c. Accumulation of zinc*

Radioactive zinc has been detected in marine fish following nuclear explosions in the Pacific area (e.g., Saiki, Okano, and Mori, 1955). Subsequently, the absorption of zinc by aquatic animals has been demonstrated by several workers. In each study, the zinc was labelled with zinc-65.

Saiki and Mori (1955) exposed clams (*Meretrix meretrix*) and carp (*Cyprinus carpio*) to low concentrations of zinc in sea water and fresh water, respectively. The test animals were cultured in zinc solutions for periods up to 22 days. Zinc was detected mainly in the gills, mantle, and viscera of clams, but 40 per cent of it was lost within two days after return to zinc-free sea water. Most of the zinc absorbed by the carp was divided equally between gills and kidney. When zinc was injected into the muscle of carp, however, most was detected in the kidneys and little in the gills.

Cairns and Scheier (1957) exposed bluegills (*Lepomis macrochirus*) to water containing 1 p.p.m. zinc. After one hour's exposure, zinc was detected in mucus covering the surface of the fish. After 24 hours, most of the zinc (and the mucus) had been precipitated to the bottom of the container, but some of the zinc had been absorbed by the fish.

Lloyd (1960) reported that two rainbow trout killed by a 20 p.p.m. solution of zinc contained respectively 7.4 and 12 parts of zinc per million parts of tissue, wet weight. The concentration of zinc measured in the whole fish was therefore only half the concentration of zinc in the water. The gills of these fish contained 63 and 60 p.p.m. zinc, wet weight — three times the concentration in solution. In another experiment, Lloyd determined by an unstated method that the concentration of zinc in the gills of two other trout, also killed by a 20 p.p.m. solution of zinc, was 1,405 and 1,265 p.p.m. respectively, dry weight. The method of drying was not reported.

Joyner (1961) studied in detail the uptake and retention of zinc by starved brown bullheads (*Ictalurus nebulosus*), during and after non-lethal treatments with zinc chloride. Groups of three fish, weighing eight grams per group, were exposed for 96 hours to 2-liter samples of

lake water (hardness 75 p.p.m. $CaCO_3$) containing initial concentrations of 0.25, 0.50, 1.0, 3.0, and 6.0 p.p.m. zinc. The fish : zinc ratios were within the range of 16,000 : 1 to 660 : 1. The actual concentrations of zinc in the solutions during exposure of the fish were not reported, but due to the high fish : zinc ratios they were probably much lower than the initial concentrations. (Compare with Cairns and Scheier's work.) The rate of uptake was first rapid and then slow in all treatments, but this may have been due to the diminishing concentrations in the solutions. The concentrations of zinc in the whole fish (wet weight) never exceeded the initial concentrations in the solutions in which the fish were immersed. Over half the total amount of zinc absorbed by the fish after 96 hours was detected in the gut and gills, with decreasing quantities in liver, kidney, skin, muscle, bone, and spleen. Bullheads exposed for 96 hours to a solution that contained initially 6 p.p.m. zinc lost 43 per cent of their total accumulated zinc after 24 hours in zinc-free lake water. At the end of a further six days, only 11 per cent more of the zinc had been lost.

Joyner and Eisler (1961) described fingerlings (*Oncorhynchus tshawytscha*) that had been exposed to a concentration of 0.2 p.p.m. zinc in lake water for 24 hours. Nearly all the zinc was retained in the fish for 63 days. Its initial location was not determined, but after a week much of it was detected in the bones. Joyner and Eisler suggested that the bone surface acted as an ion-exchange bed that readily absorbed the zinc. Later, the overgrowth of normal bone tissue in the rapidly growing fish sealed in and isolated the zinc.

Slater (1961) studied the accumulation of zinc by three species of trout: rainbow trout (*Salmo gairdneri*), cutthroat trout (*S. clarki*), and brook trout (*Salvelinus fontinalis*). Single, starved, juvenile fish were immersed for 48 hours in 200 ml samples of lake water containing 0.37 p.p.m. zinc. The fish : zinc ratio apparently ranged from 8,400 : 1 to 42,000 : 1. Unlike the bullheads in Joyner's (1961) experiments, the trout accumulated zinc at approximately the same rate for 48 hours, in spite of the high — and necessarily increasing — ratio of fish to zinc in solution. Brown trout absorbed the most zinc, and rainbow trout the least.

All the investigations reviewed in this section have shown that zinc entered fish that were exposed to zinc salts in solution. The concentrations of zinc that entered the whole fish, on a wet-weight basis, were in every case lower than the concentrations of zinc in the surrounding medium (Joyner, 1961; Lloyd, 1960). Higher concentrations were localized in gills, kidneys, and gut. However, it is still unknown how much zinc naturally occurs in fish that have not been exposed to dissolved zinc. To estimate this, a different method of zinc assay is necessary, for example polarography. Tagging with zinc-65 has shown how much zinc enters a fish that has been exposed to a solution of zinc, but it has not been used to assess the concurrent loss of zinc previously in the fish's body. The relative importance of exchange and accumulation of zinc in fish exposed to a solution of zinc have so far not been investigated.

### *d. Entry of zinc*

The possible routes by which zinc compounds may enter the bodies of aquatic animals are the gills, body surface, and alimentary canal. Jones (1939c) assessed their relative importance to freshwater fish in the following passage: "It is generally believed that the integument of the teleost fish is completely impermeable to dissolved salts, but the fact that freshwater fish normally swallow very little water, though their urine is dilute and copious, seems to imply that they absorb quantities of water through the gills and the lining of the mouth cavity as suggested by Smith (1930), though whether salts enter the body in this way is uncertain" (p. 432).

In 1963, little more can be said than that. Joyner (1961) demonstrated that when the esophagus of the brown bullhead was plugged with petroleum jelly, the fish absorbed as much zinc as untreated fish. In both groups, the gut wall contained a high concentration of zinc. Joyner believed that zinc entered the fish through both the gills and the skin. In particular, he suggested that zinc combined with mucus secreted by the skin, and was subsequently taken up by the fish from the mucus. Concerning the latter route, Cairns and Scheier (1957) detected zinc in the mucus secreted by bluegills that had been exposed to a solution

of zinc ions. Most of the zinc — and mucus — was later precipitated onto the bottom of the test tank. Saiki and Mori (1955) found that carp immersed in a zinc solution contained a large amount of zinc in the gills, but carp injected intramuscularly with zinc contained very little zinc in the gills. Thus, there is some direct evidence that zinc is not absorbed by fish through the gut, slight direct evidence that passage through the skin is unlikely, and meager indirect evidence that infiltration through the gills may be substantial. Concerning invertebrates, the only comment has been by Anderson (1948), who suggested that the resistance of *Daphnia magna* to poisons decreases at ecdysis because the exoskeleton may then be more permeable.

### *e. Theories of toxic action*

A number of theories have been proposed to explain how zinc compounds kill aquatic animals, especially fish. These are as follows:

1. Coagulation of mucus on the gills of fish causes the breakdown of certain vital processes, particularly gas exchange, nitrogenous excretion, salt balance, and circulation of the blood.
2. Cytological damage to the gills of fish causes similar breakdowns.
3. Zinc coagulates protoplasm, following its absorption into the bodies of aquatic animals.
4. Long exposure of fish to low concentrations of zinc subjects them to stress, which induces in essential organs adverse changes that result in death.

The opinions of the principal workers in the field of zinc toxicity, concerning the toxic action of zinc, will now be outlined.

Carpenter (1927, p. 390; 1930, p. 407) believed that lethal concentrations of zinc, lead, copper, and cadmium salts kill fish by suffocation induced by mucus coagulated on the gills. Carpenter did not believe that heavy-metal ions actually pentrate the body.

The same view was shared by Jones (1938, p. 406; 1939c, p. 435; and 1947a, p. 310) on the basis of his work on zinc, lead, and copper salts. He further suggested that the action of salts of the alkaline earths antagonizes the action of salts of the heavy metals by preventing the coagulation of mucus. Suffocation occurs when coagulation proceeds faster than the secretion of fresh mucus. Concerning tadpoles and *Polycelis nigra* (a planarian), Jones suggested (1939a, p. 332 and 1940a, p. 415) that heavy-metal salts penetrate the body and coagulate protoplasm either generally or selectively.

Ellis (1937, p. 401–2) proposed all of the variations of theories 1 and 2 listed above. He suggested that high concentrations of salts of the heavy metals, and also various other unspecified compounds, kill fish by anoxemia, carbon-dioxide retention, and circulatory collapse, following the clogging of the gills with precipitated mucus and direct damage to the gill cells. He further suggested that more dilute solutions of heavy-metal salts might poison the gill cells, and so render them unable to excrete chloride ions and nitrogenous wastes.

Lloyd (1960, p. 91) concluded from his work that zinc sulfate acts specifically on the gills of fish, and does not act as an internal poison. Lloyd did not consider that mucus interferes seriously with gill function, but he had no specific suggestions concerning the cause of death.

Crandall and Goodnight (1963, p. 71) suggested that the prolonged exposure of fish to low levels of zinc sulfate or other poisons subjects them to stress. This causes a hormonal imbalance which induces a variety of pathological changes. In addition, normal growth and maturation are inhibited, possibly by inadequate food intake but more probably by poor food utilization. A combination of numerous adverse changes results in general enfeeblement and ultimately in death.

Clearly, several of the opinions just outlined are contradictory. The four theories proposed at the beginning of the present section will now be evaluated in the light of our present knowledge. Finally, some generalizations will be made about the toxic action of zinc.

Information concerning the coagulation of mucus on fish gills by acutely toxic concentrations of zinc salts is conflicting (sect. IIIa). Carpenter (1927) and Jones (1938) both observed that zinc coagulated mucus on the gills and Lloyd (1960) that it did not. Only Lloyd supported his observation by histological data. Moreover, no one has effectively demonstrated

that coagulated mucus, from whatever cause, interferes in any way with any vital function of fish, although Ellis' interesting observation about the heart beat has been noted (sect. IIIb).

Only one author (Lloyd, 1960) has demonstrated by histology that cellular damage to the gills occurs when fish are exposed to a zinc salt, although three other workers (sect. IIIa) have shown that a variety of other compounds have the same effect on fish. No one has shown that gill damage causes death through breakdown of any vital gill function, although it is usual and reasonable to presume that it does. It would be interesting to know which essential function breaks down first when either the gills become damaged, or covered with coagulated mucus, or when both these changes occur together. Presumably, if the gill epithelium were sloughed off without the gills becoming clogged, gas exchange might even be facilitated, because the red blood cells would then be separated from the oxygen in solution only by the capillary walls. In this case, death might result primarily from an upset of salt balance. This idea could be readily tested by comparing the survival of poisoned fish in tapwater and in a suitable, isotonic, salt solution.

Evidence supporting the third theory is quickly disposed of: there isn't any! A succession of workers have shown that zinc is absorbed by fish and clams, both from lethal and non-lethal concentrations (sect. IIIc). However, none of them have observed any coagulated protoplasm in any aquatic animal.

The fourth theory is supported by the evidence of numerous cellular changes described by Crandall and Goodnight (sect. IIIa). These changes are similar to those occurring in fish and higher vertebrates subjected to various kinds of stress (e.g., Rasquin and Rosenbloom, 1954; Selye, 1950). The sequence of events occurring in vertebrates exposed to stress is summarized by Deevey (1959). The histological changes noted by Crandall and Goodnight correspond well to Deevey's description of the later stages of the stress syndrome. Unfortunately, Crandall and Goodnight did not extend their study to examine the interrenal tissue (which is homologous to the adrenal cortex of mammals), and the other endocrine glands. It is changes in these tissues that are believed to trigger off the widespread modifications of the type noted by Crandall and Goodnight.

Summarizing this section so far, the support for the four theories is as follows. The "coagulated-mucus" theory is possible but unproved in those situations where mucus is precipitated. The "gill-damage" theory is probable but unproved in acutely toxic concentrations of zinc. The "coagulated-protoplasm" theory is untested. The "stress" theory is probable and partly-proved where fish are exposed to chronically toxic concentrations of zinc.

From the little that is known about the toxic action of zinc compounds, three generalizations concerning fish appear fairly safe. The first is that toxic action varies with concentration. At acutely toxic concentrations, zinc appears to act primarily on the gills of fish, although it should be remembered that histological changes have not been looked for elsewhere. At chronically toxic concentrations, on the other hand, zinc causes no damage to the gills, but widespread changes occur in many other organs.

The second generalization is that the toxic action of zinc varies with life history. Fish eggs cannot die from gill damage, because they do not possess gills. Reliable information about fish eggs and fry is however entirely lacking.

The third generalization is that the toxic action of zinc is non-specific. Gill damage in adult fish is caused by a selection of unrelated compounds. Stress-induced changes are believed to be caused by a variety of adverse situations, most of them non-toxic. So far no symptoms have been recorded that would enable a toxicologist to diagnose zinc poisoning as the cause of death in any aquatic animal.

## IV. SUMMARY

Concentrations of zinc compounds that have been reported as lethal to aquatic animals have varied widely, owing to the broad range of conditions under which toxicity bioassays have been carried out, particularly the duration of exposure. In one recent study, the relationship between concentration and survival time was satisfied by Ostwald's equation, or by a modification of it.

The toxicity of zinc compounds is modified by several environmental factors. Salts of the alkaline-earth metals are antagonistic to the

action of zinc salts, and salts of certain heavy metals are synergistic in soft water. Both an increase in temperature and a reduction in dissolved oxygen concentration increase the toxicity of zinc.

The resistance of aquatic animals to zinc poisoning varies at both the individual and the species levels. The resistance of fish has been demonstrated to increase on acclimatization to sublethal concentrations of zinc, to low oxygen levels, and to hard water. Resistance may also vary with age.

Toxic concentrations of zinc compounds cause adverse changes to the morphology and physiology of fish. Acutely toxic concentrations induce cellular breakdown of the gills, and possibly the clogging of the gills with mucus. No histological changes to other organs have been noted. Concurrent with the gill changes, respiration decreases and the opercular rate increases. Chronically toxic concentrations of zinc compounds, in contrast, cause general enfeeblement and widespread histological changes to many organs, but not to gills. Growth and maturation are retarded. All the above changes have also been observed when fish are exposed to several other poisons.

Fish can detect dissolved zinc down to 10 p.p.m., and they avoid it if they can. Their behavior is similar toward several other poisons.

Fish exposed to either a toxic or a non-toxic solution of zinc absorb zinc by an unknown route, and concentrate it especially in the gills, gut, and liver. Most of the absorbed zinc is subsequently lost if the fish are returned to zinc-free water.

The mode of toxic action of zinc is uncertain. At acutely toxic concentrations it probably kills adult and juvenile fish by destroying the gill tissues. At chronically toxic levels it may induce stress that results in death. The action of zinc is undoubtedly different at different concentrations, it varies with life history, and it is non-specific.

### ADDENDUM

Since this manuscript was written, Mr. R. Lloyd has generously sent me, in advance of publication, the manuscript of a review paper presented by himself at the Third Seminar on Biological Problems in Water Pollution (Lloyd, 1962). Lloyd discusses some of the factors influencing the toxicity of the heavy metals to fish, and briefly presents a new theory concerning the mode of toxic action of the heavy metals. Substantially the same conclusions are drawn by Lloyd and myself, in the few sections of the two reviews which overlap. In addition, Lloyd describes some recent work at the Water Pollution Research Laboratory, Stevenage, U.K., which is not reviewed here. It has been demonstrated that activity decreases the resistance of fish to zinc sulfate and ammonium chloride, and that the toxicity of mixtures of poisons (including zinc) may be predicted from the known toxicity of individual compounds.

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